Cytokine and ROS Production by White Blood Cells after Exposure to Environmental Toxicants, Bacterial Analogs and Mixtures of These

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Abstract

The purpose of this study was to investigate immunomodulating effects of environmental toxicants on cytokine and ROS production in white blood cells. Immunomodulating effects can either be investigated on a healthy immune system or on an immune system during the challenge of infection. To simulate infection two bacterial analogs, LPS and Pam3CSK4, and the bacteria *Y.enterocolitica* were used.

Y.enterocolitica was used as a test candidate for the development of a whole blood model for characterization of bacteria. A concentration-dependent increase in Tnf-α production was observed after exposure to *Y.enterocolitica*. PBS should be used as a solvent for the bacteria, because cytokine production was induced by the growth medium (BHI). The volume of PBS (100µL-1mL) did not seem to affect the relative Tnf-α production pr. CFU in whole blood. The Tnf-α production in whole blood was also tested after exposure to the environmental toxicants A1242, A1254, Bisphenol A, HBCD, PCB77, PCB153, TBBPA and Triclosan. The first screening showed small but significant differences between the controls and all the toxicants tested. Different concentrations of A1254, HBCD and PCB77 were investigated further; no trends were observed. Priming with the bacterial analogs LPS (1ng/mL) and Pam3CSK4 (100ng/mL) showed no immunomodulations.

Induction of ROS-formation in human neutrophil granulocytes by exposure to A1254, Bisphenol A, HBCD, PCB153 and Triclosan were investigated with a luminol chemiluminescence assay. All of the toxicants, except Bisphenol A induced a concentration-dependent increase in ROS formation. Bisphenol A induced a reduction of the basal DMSO level of ROS. Immunomodulations of some of these responses were investigated with LPS priming or coexposure with 250 000 CFU *Y.enterocolitica*. The LPS priming seemed to suppress the normal response to the toxicants. *Y.enterocolitica* coexposed with PCB153 and Triclosan suppressed the effect of the single toxicants at higher concentrations, while A1254 and PMA seemed unaffected. Binary mixtures of Triclosan, HBCD or Bisphenol A with PMA or PCBs were also investigated and compared to predicted values by the Loewe or the Bliss model. Only mixtures between Triclosan and A1254 or HBCD were significantly different from DMSO. PCBs mixed with HBCD seemed to follow concentration addition, and the mixture with HBCD and Triclosan followed independent action more closely. The models predicted the combinatory effect of all the other mixtures poorly.

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Content

Abbreviations

A1254/A1242 – Aroclor 1254/1242

ANOVA – Analysis of variance

AUC – Area under curve

BFR – Brominated Flame Retardants

CFU – Colony-forming unit

DCHF-DA - 2′,7′-dichlorofluorescin diacetate

DMSO - Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

HBCD- Hexabromocyclododecane

IKK – IκB kinase

IL-1β – Interleukin-1β

IRAK – Interleukin-1 receptor associated kinase

LBP- LPS-binding-protein

LPS - Lipopolysaccharide

MALDI-TOF- Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry

MPO - Myeloperoxidase

NADPH oxidase - nicotinamide adenine dinucleotide phosphate-oxidase

NFκB – Nuclear transcription Factor κB

ns – not significant

OD – Optical Density

PAMPs – pathogen associated molecular patterns

Pam3CSK4 - $Pam_3Cys\text{-}Ser\text{-}(Lys)_4$

PBS - Phosphate Buffered Saline

PCB – Polychlorinated Biphenyls

PCR – Polymerase Chain Reaction

PKC – Protein kinase C

PMA - Phorbol 12-myristate 13-acetat

RNS – Reactive Nitrogen Species

ROS – Reactive Oxygen Species

SD- Standard deviation

SOD - superoxidedismutase

TBBPA – Tetrabromobisphenol A

2,3,7,8-TCDD - 2,3,7,8-tetrachlorodibenzo-p-dioxin

TLR – Toll-like receptors

Tnf- α – Tumor necrosis factor α

TIR domain – Toll/Interleukin-1 receptor domain

TRAF – TNF receptor associated factor

TRIF – TIR-domain-containing adapter-inducing interferon β

1 Introduction

1.1 Background

The air we breathe and the food we eat contain compounds that humans are not aware of, such as dust, pathogens, environmental toxicants and pollutants. Pathogens are microorganisms which cause disease; examples are bacteria (food poisoning, plague, meningitis), fungi (allergic reactions, pneumonia) and viruses (the common cold, diarrhea, chicken pox) (Parham, 2009c). The environmental pollutants that humans are exposed to might contribute to the development of illnesses such as cancer (Kriek et al., 1998), chronic obstructive pulmonary disease (COPD)(Cazzola et al., 2007) and asthma (D'Amato et al., 2005).

Bacteria, algae and spores that are present in soil or water can be transported by wind in drops of water, or on top of particles of dust or soil. Aerial dispersal is vital for many microbes in order to colonize new sites or as a part of their reproduction. Some fungi actively disperse spores in the air (Kuske, 2006). A number of these microbes may be pathogenic, as exemplified by the outbreaks of meningitis during the height of the winter dust winds in some West African regions (Sultan et al., 2005).

The types of bacteria in the air are as diverse as in soil or water (Radosevich et al., 2002; Maron et al., 2005). Bacteria are mainly divided into two groups, gram-positive and gram-negative. Gram-positive bacteria are stained purple by gram stain, while gram-negative bacteria remain unstained because they have an outer membrane with complex macromolecules such as lipopolysaccharide (LPS) and lipoprotein (Lawrence, 2000; Parham, 2009c). The outer membrane of gram-negative bacteria help them survive the host's defenses and make them more resistant to antibiotics (Campbell and Reece, 2005b). With DNA fingerprinting, Maron et al. (2005) found that 60% percent of an airborne bacterial community in rural France was gram-negative and 23% was gram-positive. Another study, by Radosevich et al. (2002) found that 50% percent of a sample from Utah were gram-positive.

Other contaminants of air are environmental toxicants. They may be organic chemicals, such as brominated flame retardants (BFR) and polychlorinated biphenyls (PCB), or inorganic chemicals such as mercury and lead. They are often bio-accumulative, persistent and may adversely affect ecosystems. These toxicants can spread from products into water, air and soil

due to chemical processes, accidents at production facilities and during transport or waste handling. Persistent chemicals may bioaccumulate through the food chain and reach harmful concentrations for wildlife and for people eating contaminated food. Studies have shown that environmental contaminants can affect the reproductive system, the nervous system, cause cancer and be mutagenic (KLIF, 2011a). There are also concerns that they might affect the immune system (Klaassen and Watkins, 2003b).

One cannot live without air and since it is a medium of transport for both environmental toxicants and bacteria, it is important to know how these contaminants affect the immune system. However, a problem, often encountered when studying immune effects of environmental toxicants, is relevant endpoints. The immune system is complex and comprised of several different organs and cells. Suppressed or activated effects are easy to spot if we are looking at the right endpoints, but what if some effects are averted from one type of immune effect to another (from inflammation to an allergic reaction)? Because of this, Holmstrup et al. (2010) define immunotoxicity as *"any modulation (activation, suppression or deviation) of immune responses by chemicals that cannot be related to the infection with a certain type of pathogen"*.

1.2 Innate Immune Response in Whole Blood

Whole blood contains plasma and cells (Fig. 1). Plasma is made up of 90% water, ions, proteins and substances transported by the blood (nutrients, waste products, hormones). The cells are platelets, erythrocytes (red blood cells) or leukocytes (white blood cells). Leukocytes can be subdivided into basophils, eosinophils, lymphocytes, neutrophils and monocytes. Their function in blood is for defense and immunity (Campbell and Reece, 2005a).

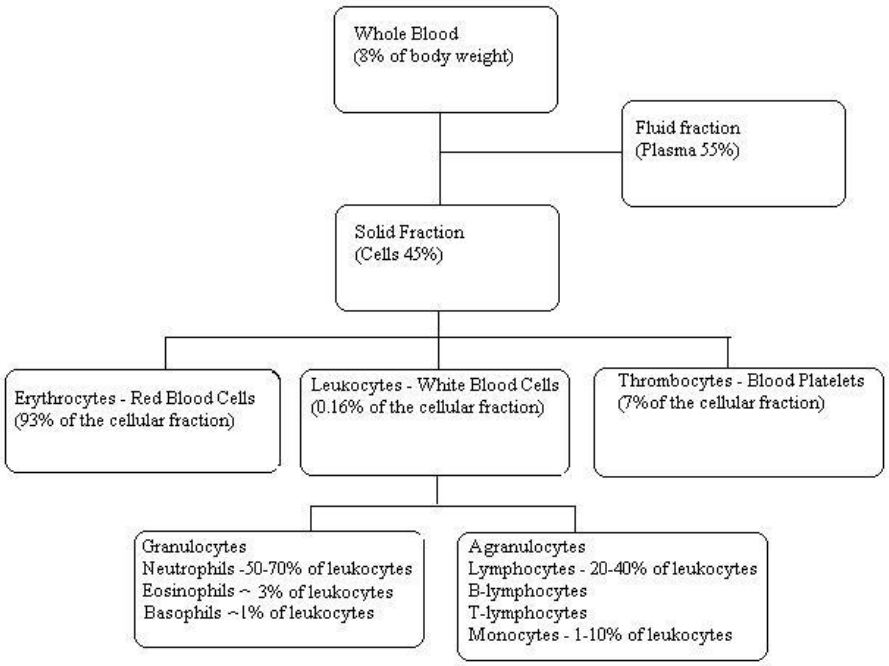


Figure 1 Constituents of Whole Blood (modified from (NetResourcesInternational, 2011))

The immune system is divided into the adaptive- and the innate immune system. The main component of the adaptive immune response is lymphocytes. They are divided into T-lymphocytes, which cause cell-mediated immunity, and B-Lymphocytes, which cause antibody-mediated immunity (Parham, 2009d). The adaptive immune response increases effectiveness towards a bacterial infection after the first infection.

The innate immune system has external and internal factors, and it acts at the onset of infection, with equal vigilance every time. The external factors are barriers that prevent pathogens from crossing the epithelia and colonizing tissues, such as skin and mucosal surfaces, and the enzymes and acidity of the gastrointestinal tracts. The internal factors consist of cells such as granulocytes and monocytes and the complement system (Parham, 2009d).

There are three types of granulocytes: eosinophils, basophils and neutrophils. Eosinophils produce chemicals that kill parasitic worms. Basophils release histamine and heparin. The cells are both involved in allergic manifestations (Sherwood, 2001). Neutrophils are the most abundant granulocytes and their effect will be described in section 1.2.2 below.

Monocytes circulate the blood for approximately three days before differentiating to macrophages or dendritic cells in lymphoid tissue. During an infection they respond to inflammation signals in the blood and move to the site of infection to mature. Macrophages are large phagocytes which ingest dead cells, bacteria or foreign materials and store it in vacuoles before disposal. Dendritic cells have similar functions, but they also act as cellular messengers that activate the adaptive immune response at the onset of an infection (Parham, 2009d).

1.2.1 Toll-like Receptors

Toll-like Receptors (TLRs) help the innate immune system to recognize pathogens such as bacteria, fungi and viruses. TLRs are mainly expressed on dendritic cells and macrophages and control activation of these antigen-presenting cells (Parham, 2009b). Monocytes and Neutrophils also express some TLRs (Wang et al., 2000). There are 10 TLRs in the human genome. These receptors recognize conserved features of microbes called pathogen associated molecular patterns (PAMPs). Each TLR recognizes several different PAMPs (Parham, 2009b).

Activated TLRs induce formation of several cytokines. One example is tumor-necrosis-factor alpha (Tnf-α) (Parham, 2009b). Tnf-α is not usually detectable in healthy individuals, but elevated serum and tissue levels are found during inflammatory and infectious conditions (Nurnberger et al., 1995; Robak et al., 1998). Another cytokine synthesized at the onset of infection is interleukin-1 beta (Il-1β), which activates lymphocytes. Both Tnf- α and IL-1β are pyrogenic, which means that they cause fever (Parham, 2009b). It is mainly monocytes that produce cytokines in whole blood (Wang et al., 2000). The cytokine cascades for viruses and gram-negative and -positive bacteria are similar, even though they are initiated by different TLRs (Parham, 2009b).

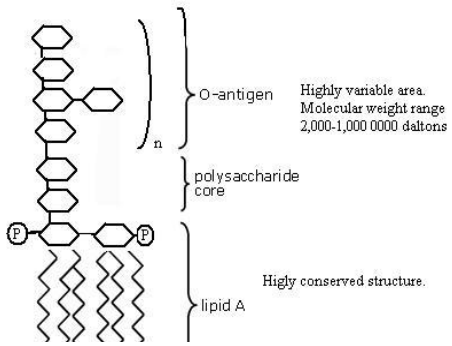


Figure 2 Lipopolysaccharide modified from ((Spectral Diagnostic Incorporated, 2006))

LPS is an endotoxin, with a polysaccharide region (o-antigen) that varies somewhat in different gram-negative bacteria, but they have a similar structure and they are all pyrogenic (Fig. 2). LipidA is the most conserved part of LPS. This region often activates

TLR4, though some lipid A structures activate TLR2 instead (Bainbridge and Darveau, 2001; Pridmore et al., 2001; Werts et al., 2001).

All TLRs except TLR3 activates the MyD88 dependent pathway (Fig.3) which cause inflammatory cytokine production. TLR4 and TLR3 activate the MyD88 independent pathway which causes production of interferons (Fig. 3) (Kumar et al., 2009).

In the Myd88 dependent pathway, LPS-binding-Protein (LBP) binds to LPS in the blood. LBP delivers LPS to CD14 on the cell membrane. CD14 interacts with MD2-TLR4, which has a Toll/Interleukin-1 receptor (TIR) domain in the cytoplasm. TIR recruits MyD88, which phosphorylates interleukin-1 receptor-associated kinase 4 (IRAK4). Then TNF receptor associated factor (TRAF) 6 is phosphorylated and a kinase cascade begins. The cascade ends with phosphorylation of IκB kinase (IKK) (Schumann et al., 1990; Wright et al., 1990; Chow et al., 1999; Hoshino et al., 1999; Yang et al., 1999; Anderson, 2000; Beutler, 2000; Parham, 2009b).

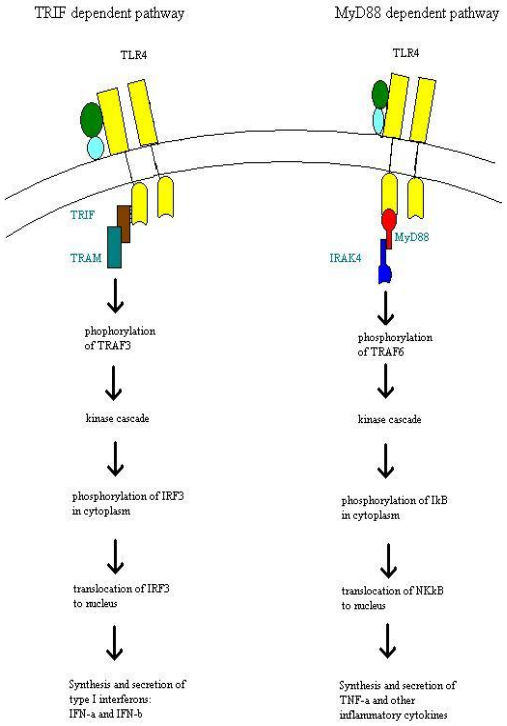


Figure 3 Figure 4TLR4 signaling by the TRIF and MyD88 pathways modified from Parham (2009a).

Nuclear transcription factor κB (NFκB) is bound by its inhibitor IκB which prevents it from entering the nucleus. IKK phosphorylates IκB and this induces the release of NFκB from the complex. NFκB activates genes encoding inflammatory cytokines like Tnf-α and Il-1β (Parham, 2009b).

Alternatively MD2 recruits TIR-domain-containing adapter-inducing interferon-β TRIF, which in turn recruits TRIF-related adaptor molecule TRAM. TRAF 3 is then phosphorylated and a kinase cascade is started. The cascade ends with interferon regulatory factor (IRF)3 phosphorylation. IRF3 enters the nucleus and activates genes encoding type I interferons (IFN-α and IFN-β) (Parham, 2009b).

The cytokine cascade activated by TLRs is both pro- and anti-inflammatory. An inflammatory response is beneficial at first, because it helps to clear the infectious agent. Prolonged

inflammation is harmful because it may cause host toxicity and tissue damage (Zeytun et al., 2010). An example is if a local infection becomes systemic, which causes the macrophages in the liver and spleen to start to secrete Tnf-α into the blood stream. If this goes unchecked it can cause organ failure and death. This is known as septic shock (Parham, 2009c). In other cases an inappropriate immune response may give rise to a prolonged and damaging inflammatory response like the chronic inflammatory disease rheumatoid arthritis, which causes severe pain in joints. Rheumatoid arthritis has been successfully treated with Tnf-blocking agents, which indicates that Tnf-α is involved in the inflammation process (Bradley, 2008).

In studies of septic shock *in vitro* a whole blood model is often used with LPS as the instigator of the cytokine cascades. This model cannot be directly transferred to *in vivo* reactions because tissue macrophages also contribute greatly to the reaction in humans (Dorresteijn et al., 2010), but it is still useful for investigation of monocyte cytokine production. It is more cost efficient than isolating monocytes during the experiments, it gives the monocytes a more relevant environment, and pre-activation during monocyte isolation is avoided. Neutrophils may also produce cytokines, but their capacity for production is much lower than the monocytes' (Damsgaard et al., 2009). Many white blood cells of the innate immune system have TLRs which are activated by PAMPs (Wang et al., 2000; Parham, 2009b). The whole blood model can, with detection of cytokines or interferons, give an indication of immunomodulations after exposure to environmental toxicants or a mixture of environmental toxicants and known PAMPs.

1.2.2 Neutrophil Granulocytes

Of the granulocytes, neutrophils are the most abundant and the first line of defense after a bacterial invasion. They circulate the blood and migrate through tissue. Depending on their actions they can live from seven hours to two days (Quinn and Gauss, 2004). They are attracted to the cytokines and chemokines produced in response to TLR activation (Knapp, 2010). When entering a contaminated site they engulf invading microorganisms into a phagosome and produce several cytotoxic agents to kill the organism (Hampton et al., 1998). During the phagocytosis of the intruding organism the neutrophil induces production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in a process called

respiratory burst (Freitas et al., 2009). This is harmful for the invading pathogens, but overproduction of ROS/RNS can also be harmful to the host (Conner and Grisham, 1996).

Examples of reactive oxygen species are hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet -}$), hypochlorite (OCl^-) and the hydroxy radical ($OH^{\bullet}$). The superoxide anion ($O_2^{\bullet -}$) is formed in many auto-oxidation reactions and by the electron transport chain. Superoxide anion is transformed to hydrogen peroxide (H_2O_2) by the enzyme superoxide dismutase (SOD). The Fenton reaction creates $OH^{\bullet}$ from a reaction between H_2O_2 and Fe(II). $OH^{\bullet}$ reacts with virtually all biomolecules and is perhaps the most harmful oxygen radical.

Reactive oxygen species are potentially toxic to the cells. They can initiate or enhance an inflammation response by upregulation of genes; for example, NF-κB (Conner and Grisham, 1996). They can react with lipids, proteins, nucleic acids and other molecules and affect these molecules' normal structure or function. Initiation of free radical-induced lipid peroxidation can result in chain reactions leading to the destruction of the cellular membrane (Myhre, 2001). They have been linked to neurodegenerative disorders like Parkinson's, Huntington's and Alzheimer's (Fonnum, 1998). Since ROS/RNS are physiologically useful in small amounts there are no systems in the cells that remove them completely (Barja, 1993). They, can for example, function as mediators of signals between membrane receptors and target proteins. An oxidative attack on a protein induces either a loss of function, a gain of function, or a switch to a different function (Droge, 2002).

The respiratory burst in neutrophils is caused by the NADPH oxidase system, which is activated by a phosphorylation cascade. The NADPH oxidase system is a membrane bound enzyme with several protein subunits that catalyzes the production of superoxide radical from oxygen (Henderson and Chappel, 1996; Hampton et al., 1998; Babior, 2000; Babior, 2002; Quinn and Gauss, 2004; Freitas et al., 2009). The superoxide radical is relatively non-reactive in the cell, but rapidly changes to H_2O_2 at low pH or by superoxide dismutase (SOD) (Hampton et al., 1998). In neutrophils most of the H_2O_2 produced by NADPH is used by myeloperoxidase (MPO) to form HOCl, which is a potent bactericidal oxidant (Hampton et al., 1998).

1.2.3 Methods for Detection of Cytokines and ROS

Cytokines and ROS production can be induced by chemicals as well as by pathogens. This means that the adaptive immune response might be stimulated from several sources at the same time and the actual response to pathogens might be upregulated, suppressed or diverted to another reaction (Holmstrup et al., 2010). Because of this, it is important to investigate how environmental toxicants affect cytokine and ROS production. Several methods have been developed that can be used to detect cytokines and ROS.

ELISA -Enzyme Linked Immunosorbent Assay

The ELISA is a specific and highly sensitive method for quantitative detection of cytokines or other chemicals in solution. A specific antibody is coated on a microtiter plate and the cytokine of interest will attach itself to the antibody. Then a second antibody, used for detection, binds on a different epitope on the cytokine. The detection antibody is labeled with biotin, which allows subsequent binding of a Streptavidin-conjugated enzyme. Any unbound reagents are washed away. Finally a substrate is added, causing a color reaction to develop that is proportional to the amount of cytokine bound. The concentration of cytokine is determined by comparison with a standard curve with known concentrations of cytokine. The detection limits for cytokine ELISAs are commonly in the lower picogram/ml range (Mabtech AB, 2011).

Probes for Detection of Reactive Oxygen Species

Formation of reactive oxygen species can in principle be measured in several ways *in vitro*. Among these are fluorometric probes (HE, Amplex red, DCFH etc.), colorometric probes (TMB, Cytochrome C) and chemiluminometric probes (Luminol, Lucigenin). Several of these have limitations such as low sensitivity and selectivity (Cytochrome C), underestimation of ROS production (HE) and dependence on HRP activity may occur (Amplex red, DCFH). (Freitas et al., 2009). Lucigenin, which only measures extracellular $O_2^{\bullet-}$ (Kopprasch et al., 2003; Pavelkova and Kubala, 2004) is highly specific (Freitas et al., 2009), but since it only measures $O_2^{\bullet-}$ it should be used together with other probes to get a complete picture of ROS production.

The luminol probe, used in this study, was introduced in 1976 by Allen and Loos. Luminol reacts with ROS to produce an excited aminophtalate anion that emits light when returning to ground state (Briheim et al., 1984; Albrecht and Jungi, 1993; Lundqvist and Dahlgren, 1996; Faldt et al., 1999). Caldefie-Chèzet et al. (2002) concluded that Luminol only detects intracellular ROS production in an experiment where they compare different probes. This is not the overall agreement, as presented in the review by Freitas et al. in 2009. Luminol detects several ROS ($O_2^{\bullet-}$, H_2O_2, $HO^{\bullet}$, HOCl) and RNS ($^{\bullet}NO$, $ONOO^-$) both inside and outside of the cell. It is dependent on myeloperoxidase (MPO) and regarded as sensitive (Freitas et al., 2009).

1.3 Environmental Toxicants

1.3.1 Brominated Flame Retardants

Brominated flame retardants (BFRs) are chemicals used to prevent fires in materials. They interrupt the free radical chain created in the gas phase of the combustion process, thereby preventing or delaying the onset of ignition and slowing the rate of burning after ignition (Morose, 2006). They can be additives in the materials, in which case they can leak directly into the environment, or they can be chemically bound to the materials, in which case they can leak when the material deteriorates or in cases where there are leftovers that do not react in the production process. They can be found in domestic products like electronic equipment, upholstered furniture, interior textiles, automobile interior textiles, house walls, cellars and roofs (de Wit, 2002). Production of brominated flame retardants was approximately 300,000 tons in 2001 (Morose, 2006). Some of these BFRs have been detected in environmental samples (de Wit, 2002).

Hexabromocyclododecan (HBCD)

HBCD, a brominated aliphatic cyclic hydrocarbon, is used as a an additive flame retardant in plastics, textiles and insulation (de Wit, 2002). Annual production of HBCD is approximately 23,000 tons (Persistent Organic Pollutants Review Committee, 2011). It is bioaccumulative and the concentration of HBCD increases along the food chain (Law et al., 2006). Humans are exposed mainly through diet, especially fish (Thomsen et al., 2008; Schecter et al., 2010), but

also through inhalation of dust particles (Thomsen et al., 2007; Roosens et al., 2009) and dermal contact with materials treated with HBCD (Thomsen et al., 2007).

HBCD has been shown in laboratory animals to be a hepatic enzyme inducer (Hamers et al., 2006), a developmental neurotoxicant (Eriksson et al., 2006), and an endocrine disruptor (van der Ven et al., 2006). HBCD also has an effect on dopamine and glutamate uptake in rat brain synaptosomes (Mariussen and Fonnum, 2003).

In vitro HBCD affects natural killer cells' lytic function and ATP levels at 10μM. Even a short exposure for one hour caused a progressive loss of lytic function over a six-day period, indicating an immunotoxic potential. This could affect a virus infection, since natural killer cells are important in recognizing and destroying virus infected cells (Hinkson and Whalen, 2009).

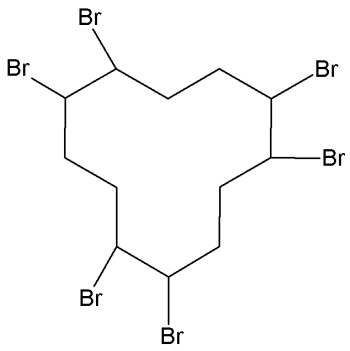


Figure 5 HBCD

Tetrabromobisphenol A (TBBPA)

TBBPA is a brominated flame retardant and is mainly used in epoxy resins of printed circuit boards and in other industrial materials (de Wit, 2002). TBBPA is used in large quantities with an annual production of approximately 200,000 tons (Morose, 2006). It chemically binds to the plastics and is not as easily released into the environment as other BFRs like HBCD. TBBPA is found in people exposed to TBBPA at work (Thomsen et al., 2001) and even in individuals with no direct occupational exposure (Thomsen et al., 2002; Hayama et al., 2004).

Acute oral toxicity is low in laboratory animals (Darnerud, 2003). The lowest dose known to cause toxicity is 200mg/kg bodyweight causing nephrotoxicity in newborn rats (Kitamura et al., 2002; Fukuda et al., 2004). TBBPA is shown to affect dopamine and glutamate uptake in rat brain synaptosomes, cause ROS formation, induce cell death, calcium influx and elevation

of extracellular glutamate in rat cerebellar granule cells *in vitro* (Mariussen and Fonnum, 2003; Reistad et al., 2007). TBBPA has a molecular structure similar to that of the thyroid hormone thyroxine (de Wit, 2002). This leads to concerns that TBBPA may interfere with the thyroid hormone system (Meerts et al., 2000; Kitamura et al., 2002).

Watanabe et al. (2010) found that TBBPA has an effect on the immune response to respiratory syncytial virus infection in mice. Pullen et al. (2003) found that TBBPA may act as an immunotoxic compound by specifically inhibiting the expression of CD25. These experiments were done on splenocytes from mice. Reistad et al. (2005) found that TBBPA caused ROS formation in human granulocytes by activating respiratory burst through NADPH oxidase.

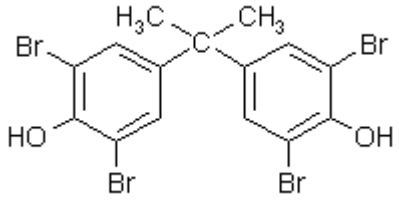


Figure 6 Tetrabromobisphenol A

1.3.2 Polychlorinated Biphenyls

PCBs were introduced in 1929 in applications like cooling material in transformers and other electrical devices, hydraulic fluids and additives in different types of paint and glue (KLIF, 2011b). Until 1984 about 1.2 million tons of PCBs had been produced worldwide and 20-30% of this has been released into the environment through burning, evaporation, leaks and dumping (Tanabe, 1988). Due to their high lipophilicity and structural stability, PCBs are persistent environmental pollutants and accumulate in higher trophic levels throughout the food chain (Tanabe, 1988; Fischer et al., 1998). In 1980, Norway followed other industrial countries and prohibited new use of PCBs (KLIF, 2011b). PCBs are still found in human or animal tissue throughout the world (Colborn et al., 1993; Kvalem et al., 2009).

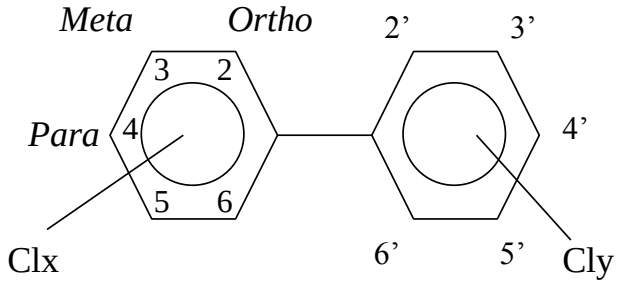


Figure 7 General PCB structure

The PCBs are a family of halogenated aromatic hydrocarbons with 209 possible congeners (Fig. 5). They are often divided into three different groups; the coplanar with no chlorine substitution in the ortho position; the non-coplanar with two or more ortho substitutions; and the mono ortho substituted PCBs, which may have a coplanar configuration some of the time. Coplanar PCBs can bind to the aryl hydrocarbon receptor (Ah-receptor). They mimic the toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD), which also binds to the Ah-receptor (Safe, 1994).

There are epidemiological studies of humans which show that PCBs may have an effect on neurological development and learning abilities (Tilson et al., 1990; Seegal, 1996; Tilson and Kodavanti, 1998; Canzoniero et al., 2006). These studies also indicate that PCBs cause skin-disorders (Meigs et al., 1954; Swanson et al., 1995), cancer (Sinks et al., 1992; Swanson et al., 1995) and reproductive and developmental abnormalities (Taylor et al., 1989; Swanson et al., 1995). Studies done with Aroclors, which are commercial PCB mixtures, showed that PCBs cause reproductive disorders on rabbit embryos (Seiler et al., 1994). Studies on other animals with Aroclors have shown that they can be estrogenic, thyrotoxic, liver enzyme inducers and immune suppressing (Harris et al., 1993; Cooke et al., 1996; Omara et al., 1997). The general opinion seems to be that ortho-substituted PCBs are primarily the cause of the neurotoxic effects observed in Aroclor mixtures (Seegal et al., 1990; Seegal et al., 1991a; Seegal et al., 1991b; Shain et al., 1991; Mariussen et al., 2002; Mariussen and Fonnum, 2006).

Ortho-chlorinated PCBs are known to activate neutrophils. Ganey et al. (1993) exposed human and rat neutrophils to the PCB mixture Aroclor 1242. The neutrophils induced formation of $O_2^{-\bullet}$ and secreted lysosomal products. Studies with single PCB-congeners have shown that the effect is attributed to the non-coplanar ortho-chlorinated PCBs (Ganey et al., 1993; Brown and Ganey, 1995; Tithof et al., 1995; Voie et al., 1998).

There are three pathways that have been shown to be necessary for activation of neutrophils by PCBs; release of intracellular Ca^{2+}(Brown and Ganey, 1995; Voie and Fonnum, 1998), possibly by activation of phospholipase C and/or D (Voie et al., 1998); tyrosine kinase activation (Tithof et al., 1997); and arachidonic acid released by activation of phospholipase A_2, which in turn activates the NADPH oxidase system (Henderson et al., 1993; Dana et al., 1994; Tithof et al., 1996; Fischer et al., 1998).

PCB 77, a coplanar PCB, has shown a potential to induce the expression of proinflammatory cytokines, including IL-6 and Tnf-α *in vitro* with human umbilical vascular endothelial cells (HUVEC). PCB was also observed to alter insulin-activated signaling by Tnf-α secretion (Wang et al., 2010).

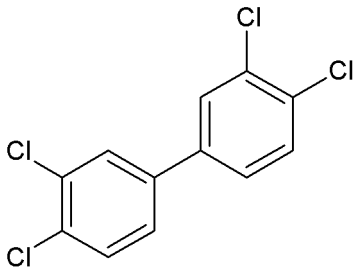


Figure 8 PCB77

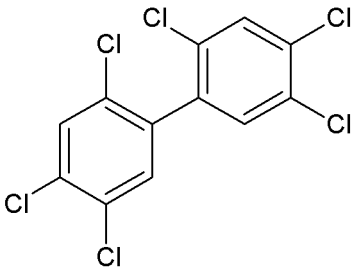


Figure 9 PCB153

1.3.3 Bisphenol A

Bisphenol A is a monomer used in production of polycarbonate plastics used in food and beverage packing. Bisphenol A is also used to make resins that line beverage cans (Talsness et al., 2009) and in dental composites (ACC, 2011). In 2002, approximately 2.8 million tons of Bisphenol A was produced globally (ACC, 2011). Bisphenol A is listed as an endocrine disruptor (Feldman, 1997; Sonnenschein and Soto, 1998). It shows estrogenic effect at high doses. Estrogens are often immunomodulating as well as reproductive agents (Kittas and Henry, 1980; Luster et al., 1984). As a result of several animal studies, concerns have been raised that Bisphenol A may affect the nervous, reproductive and immune systems both as a developmental toxicant and when exposed as adult (Richter et al., 2007).

Bisphenol A has been reported to modulate immune function at doses between 2.5 and 30 μg/(kg day), including patterns of cytokine and antibody production, response to infection and autoimmune disease progression in mice (Sawai et al., 2003; Yoshino et al., 2003). It has also

been shown that Bisphenol A exposure is associated with modulation of innate immune system cell function (Sugita-Konishi et al., 2003). Yoshino et.al (2004) reported that prenatal exposure to Bisphenol A up-regulates immune responses, including T-lymphocyte responses, in mice. Reistad et al. (2005) found a small dose-response dependent increase in ROS formation with a DCFH-DA probe on human granulocytes, but not with a lucigenin probe, indicating that the ROS formation was intracellular.

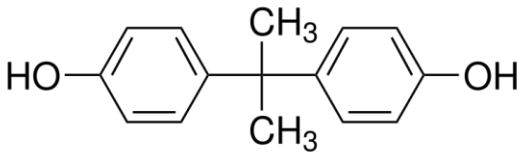


Figure 10 Bisphenol A

1.3.4 Triclosan

Triclosan adversely affects most gram-negative and gram-positive bacteria. It is used in personal care products like toothpaste, cosmetics, household items, medical devices, and in clinical settings where its antimicrobial activity is necessary (Jones et al., 2000). In Europe, 450 ton Triclosan were used in 2006 (SCCS, 2009). It is likely that many humans are exposed to Triclosan throughout their entire lifetime. It has been detected in the environment and even in human plasma at concentrations of 0.1-8.1 ng/mL. Individuals who used products containing Triclosan had elevated levels of Triclosan in their body fluids (milk and plasma) (Hovander et al., 2002; Sandborgh-Englund et al., 2006).

Triclosan disrupts membrane activity in bacterial cell membrane without causing leakage of intracellular components (Villalain et al., 2001; Guillen et al., 2004). Triclosan is also an inhibitor of the enoyl-reductase of type II fatty acid synthase involved in the bacterial lipid biosynthesis (Levy et al., 1999; Stewart et al., 1999; Ward et al., 1999; Heath et al., 2000).

Triclosan possesses intrinsic estrogenic and androgenic activity (Gee et al., 2008). There have been studies to check for reproductive toxicity, genotoxicity, carcinogenicity or chronic toxicity, but nothing significant has been reported (Fang et al., 2010). In an *ex vivo* whole blood model, Triclosan (0.5µg/mL) has shown broad anti-inflammatory activity by suppression of Tnf-α production (Barros et al., 2010).

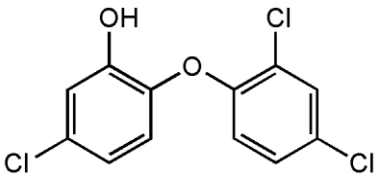


Figure 11 Triclosan

1.4 Mixture Effect Models

Environmental toxicants usually appear in mixtures. Little is known about combinatorial toxicity of environmental pollutants, and the fact that these pollutants may have additive or synergistic effects is therefore of great concern.

There are two accepted models for the study of mixture effects - independent action and concentration addition. Independent action is common in research on multiple drug-exposure (Berenbaum, 1985) and in risk management recommendations (Altenburger et al., 2000). Concentration addition is often used in aquatic toxicology and is said to be well suited for the prediction of nonspecifically acting compounds like organic non-reactive chemicals (Konemann, 1980; Konemann, 1981; Hermens et al., 1984).

Bliss (Independent action)

This model was proposed by Bliss in 1939, and assumes that each chemical has a different mode of action. This model is based on probability and claims that the effect of two compounds in a mixture is equal to the sum of the effect of each of the compounds minus the product of the effects of each compound. Effects (E) are defined as fractions of maximum effect.

$$E(x,y) = E(x) + E(y) - (E(x)*E(y)) \quad (1)$$

Independent action often gives a good prediction of the effect of different compounds in mixtures as long as they all are above their no observed effect level (NOEC) and reacts independently. The model will, however, often underestimate a mixture effect if the compounds are under the NOEC and reacts similar to concentration addition as described below (Rajapakse et al., 2002).

Loewe (Concentration addition)

This model was proposed by Loewe and Muischnek in 1926, and assumes that each chemical has the same mode of action towards a cellular target, but might have different potencies. Chemicals in a mixture at concentrations below NOEC will be able to add to the effect of the mixture. As long as the equation (2) holds for an effect X, the concentration addition holds. If the sum is higher than one, there might be some sort of antagonistic relationship, and if the sum is lower there might be a synergistic relationship (Berenbaum, 1985; Rajapakse et al., 2001).

$d_a/EC_a + d_b/EC_b = 1$ (Andersen et al., 2009)

or more generally: $\Sigma\ (c_i/ECx_i) = 1$ (Berenbaum, 1985; Rajapakse et al., 2001) (2)

d_a= concentration of substance a that together with substance b exerts a given effect = $EC_{mix}{*}p_a$

EC_a = the dose necessary for substance a to give the same effect as substance a and b together (EC_{mix}).

p_a = the ratio concentration of substance a in the mixture.

EC_{mix} = the effective concentration of the mixture.

$EC_{mix}{*}p_a /EC_a + EC_{mix}{*}p_b /EC_b = 1 \rightarrow EC_{mix} = 1/ ((p_a/EC_a) + (p_b/EC_b))$ (3)

This equation which was developed from an article by Rajapakse (2001), was used to calculate the predicted effect of mixtures.

1.5 Object of Investigation

The purpose of this study is to look for modulations of the immune response. Environmental toxicants are suspected to have immunomodulating effects. Some have shown effects on cytokine production *in vitro* (PCB77 and Triclosan) (Barros et al., 2010; Wang et al., 2010); others like Bisphenol A (Reistad et al., 2005) and ortho-chlorinated PCBs (Voie et al., 1998) have shown effects on ROS formation in human neutrophil granulocytes.

It is important to know how environmental pollutants affect a healthy immune system, but it is equally important to know how they interact with pathogens that cause disease. Will the immune response to infection be affected by environmental toxicants present? To be able to investigate this, it is, in addition, necessary to know how pathogens affect the immune response. It is also important to find out if the environmental toxicants interact, because they

often occur in the same environment. For risk assessment purposes it is therefore necessary to decide if they should be investigated separately or grouped according to their effects.

The objects of investigation in this thesis are:

- To develop a whole blood model to use on characterization of bacteria.
- To investigate environmental toxicants' effect on cytokine production of healthy cells and on cells fighting an infection in whole blood.
- To investigate the effect of environmental toxicants on ROS production in human neutrophil granulocytes.
- To investigate the immune modulating effects on ROS production in human neutrophil granulocytes when exposed to environmental toxicants and bacteria or a bacteria-analog.
- To investigate the effects of binary mixtures of environmental toxicants on ROS production in human neutrophil granulocytes to test if the Loewe- or the Bliss model predicts the result.

2 Materials and Methods

2.1 Chemicals

Table 1 Chemicals used in these experiments.

Chemicals	Producers
A1254	Accustandard Inc. (New Haven, USA)
A1242	Accustandard Inc. (New Haven, USA)
Agar	Merck (Darmstadt, Germany)
α-Cyano-4-hydroxycinnamic acid (CHCA)	Bruker Daltonics (Germany)
Brain Heart Infusion (BHI)	Oxoid Ltd. (Basingstock, England)
Bisphenol A	Sigma-Aldrich Co. (Meriden, USA)
9mL Vacuette with K_3EDTA	Greiner bio-one GmbH (Kremsmünseter. Austria)
9mL Vacuette with LithiumHeparin	Greiner bio-one GmbH (Kremsmünseter. Austria)
$CaCl_2$	Merck (Darmstadt, Germany)
Dextran	Pharmacia Fine Chemicals (Uppsala, Sweden)
Dimethylsulfoksid (DMSO)	Sigma-Aldrich Co. (Meriden, USA)
Forward primer 933F16S	Invitrogen Corporation (Camarillo, USA)
d-Glucose	Merck (Darmstadt, Germany)
KCl	Merck (Darmstadt, Germany)
KH_2PO_4	Merck (Darmstadt, Germany)
LPS Escherichia coli 0111:B4	Sigma-Aldrich (St.Louis, USA)
Luminol	Sigma-Aldrich (St.Louis, USA)
Lymphoprep	Axis-Shield PoC AS (Oslo, Norway)
Heksabromcyclododekan (HBCD)	Promochem GmbH (Germany)
H_2SO_4	Merck (Darmstadt, Germany)
$MgCl_2$	Merck (Darmstadt, Germany)
$MgSO_4$	Merck (Darmstadt, Germany)
NaCl	Merck (Darmstadt, Germany)
$NaHCO_3$	Merck (Darmstadt, Germany)
Na_2HPO_4	Merck (Darmstadt, Germany)
NH_4Cl	Merck (Darmstadt, Germany)
N-[2-hydroxyethyl] piperazine-N'-[ethansulphonic acid] (HEPES)	Sigma-Aldrich (St.Louis, USA)
PCB 153	Accustandard Inc. (New Haven, USA)
PCB 77	Promochem GmbH (Germany)
Phosphate Buffered Saline PBS	Sigma-Aldrich (St.Louis, USA)
Pam3CSK4	InvivoGen (San Diego, USA)
Phorbol 12myristate-13-acetate (PMA)	Sigma-Aldrich (St.Louis, USA)
Reverse Primer 1387R16S	Invitrogen Corporation (Camarillo, USA)
SYBR green mix I	Roche Diagnostic (Germany)
Tetrabromobisphenol A (TBBPA)	Larodan Fine Chemicals AB (Malmö, Sweden)
Triclosan	Dr. Ehrenstorfer GmbH (Augsburg, Germany)

2.2 Whole Blood Experiments

2.2.1 Preparation of *Y.enterocolitica*

Y.enterocolitica (ATCC, Chicago, USA) is a gram-negative bacteria (Brubaker, 1991) and was used as an example for real-life infection when developing the whole blood model for bacteria. These bacteria were chosen because they have LPS similar to other gram-negative bacteria (Mandel et al., 2005), and were therefore expected to produce Tnf-α. They grow in temperatures ranging from 5 to 42 ºC (Brubaker, 1991). In our laboratory they grow on brain-heart-infusion medium at 30ºC (ATCC, 2010).

Y.enterocolitica was transferred from a stock solution (still frozen) by a small inoculation spatula and spread on a Brain Heart Infusion (BHI) agar plate. Then it was grown overnight at 30ºC. To maintain fresh bacteria, a colony was re-inoculated every week. In cases of changed morphology (transparency, colour, size), a new inoculation from the stock solution was prepared as above. One colony of *Y.enterocolitica* was picked from a culture with fresh bacteria and transferred to 50mL BHI medium. The culture was rotated at 200 rpm and incubated overnight at 30ºC. Then the culture was diluted with medium to OD=0.1 (optical density at λ=600nm). OD was measured each following hour while the bacteria grew, still rotated at 200 rpm in 30ºC. At the same time points as OD was measured, dilutions (10^{-1}–10^{-7}) of the solution were made in PBS and plated out on BHI-agar, so that colony forming units (CFU) could be counted the next day. This was done with 3 parallels for each dilution at each time point.

These experiments were done to calculate the growth rate of *Y.enterocolitica* in BHI. This was used in later experiments to calculate how much culture to use to get the amount of *Y.enterocolitica* needed for the experiments. Log(OD) versus log(CFU) is supposed to be a linear slope in the exponential growth phase of bacteria (Maddigan and Martinko, 2006a). With linear regression, formula 4 was used to predict the amount of bacteria in the culture (see appendix II.i).

$$\text{Log CFU} = 0.9111 * \text{Log(OD)} + 8.6042 \quad (4)$$

Preparation of Bacteria Solution to the Whole Blood Experiment:

One colony of *Y.enterocolitica* was picked from a culture with fresh bacteria and transferred to 50mL BHI-medium. The culture was rotated at 200 rpm and incubated overnight at 30ºC. The next morning the culture was diluted with BHI-medium to OD=0.1. Then it grew for about 3 hours until OD was approximately 0.6. Then three different amounts of *Y.enterocolitica* (10^6, 10^7, 10^8) were mixed with different amounts of BHI (1, 0.5, 0.2, 0.1mL) and 1mL blood. This was done to investigate how the medium and different concentrations of bacteria affected cytokine production in whole blood. These experiments indicated that the medium could have a strong effect on the results. The experiment was repeated, but this time the bacteria was washed (*2) and dissolved in PBS.

LPS were used as a gram-negative bacterial analog. LPS from *E.coli* can induce pro-inflammatory responses like macrophage-, monocyte- and mast cell production of cytokines. These cytokines include; IL-1β, Tnf-α, IL-6, IFN-γ, IL-12, IL-13 and IL-8. (Morrison and Ryan, 1979; Kluger, 1991; Van Zee et al., 1991; Klir et al., 1993; Roth et al., 1993; Jansky et al., 1995; Wang et al., 2000; Supajatura et al., 2002; Madianos et al., 2005; Moller et al., 2005; Andersson and Sundler, 2006).

Pam3CSK4 was used as a gram-positive bacterial analog. It is a synthetic tripalmitoylated lipopeptid which functions like the acylated amino terminus of bacterial lipoproteins. Pam3CSK4 binds to a TLR2/TLR1-dimer and activates NF-κB (Aliprantis et al., 1999; Ozinsky et al., 2000). TLR2 is important in gram-positive bacteria recognition (Takeuchi et al., 1999).

2.2.2 Preparation of Whole Blood

The whole blood model is a useful tool to investigate induction of cytokines on a mixed white blood cell population. Studies have shown that the leukocyte counts in whole blood and the viability of the cells are very high, even after 24 hours without supply of oxygen. In the sealed plastic container, oxygen consumption decreases slowly over time, which indicates that metabolic activity goes down during the experiments (Wang et al., 2000).

Blood from male volunteers age 23-55 was used in all experiments. The blood was heparinized in the vacuettes used to collect blood from the donors. Each donation of blood was between 40 and 100 mL. The cell count was measured for each individual donor at each

donation with an ADVIA60 hematology system. Whole blood (1-2mL) was divided into bottles and mixed with the bacteria solutions or environmental toxicants. The blood was slowly rotated at 37ºC for six or 24 hours.

Plasma was removed from the whole blood by centrifugation in 15 minutes at 2000*g*. The plasma was frozen and stored for later analyses with ELISA according to the protocol provided by the manufacturer. We tested for known pyrogenic cytokines Tnf-α (Invitrogen Corporation, Camarillo, USA) and IL-1β (R&D systems Inc., Minneapolis, USA). The detection limit was 31.25pg/mL for Tnf-α and 7.8pg/mL for IL-1β. The reagents and antibodies used were from the manufacturers of the ELISA kits, except Coating Buffer A (Tnf-α) and stop solution (Il-1β) (see appendix I.ii).

Identification of Bacteria

Polymerase Chain Reaction (PCR)

The PCR is an enzymatic amplification of a specific section of double stranded DNA using DNA polymerase. A primer pair flanking the DNA fragment on the 3´ends specifies the amplification target sequence. DNA polymerase synthesizes a new DNA strand, in the 5´direction, using the primed DNA. The PCR reaction consists of three cyclic steps: denaturation, annealing and extension (see table 3)(Sanger et al., 1977). After the cycles have been completed the sequence can be analyzed. If it has several variable parts (not conserved gene sequences) the copied DNA can be used to identify the bacteria the DNA came from (Maddigan and Martinko, 2006b)

One colony from BHI-agar plate with *Y.enterocolitica* was diluted in 500µL dH_2O and boiled at a 100ºC for 10 minutes, to destroy the membrane so that the DNA was accessible in the solution (DNA solution). A master mixture with primers and Cybergreen mix I was made and 18µL were transferred to each well (table3). Then 2 µL of DNA solution was transferred into the appropriate wells. The primers used should give products with v6, v7 and v8, three variable parts of the conserved 16S gene. Afterwards the plate was sealed with a plastic strip and quick-spun. Then it was placed in the PCR machine (LightCycler 480, Roche Diagnostic, Germany) following the cycles described in table 3.

Table 2 The recipe used for PCR.

Reagent	Quantity in each well	Master Mixture
Enzyme mix (SYBR Green I*)	10 µL	100
Forward primer 933F16S (5 µM)	4 µL	40
Reverse Primer 1387R16S (5 µM)	4 µL	40
DNA solution:	2 µL	0

***consists of FastStart Taq DNA Polymerase, reaction buffer, cNTP mix (whit dUTP instead of dTTP), SYBR Green I dye, and $MgCl_2$.**

Table 3 PCR-program

Analysis	Temperature	Cycles	Time
Denaturation	95ºC	1	5 min
PCR:			
Denaturation	95 ºC	32	10sec
Annealing	58 ºC	32	10sec
Extension	72 ºC	32	20sec
Final Extension	72 ºC	1	5 min

The double-stranded DNA is separated by high temperature (>90^{o}C) during denaturation. The temperature is lowered to between 50-60^{o}C for annealing between single DNA strand and primer. During the extension phase, a DNA polymerase synthesizes a new DNA strand using the primed DNA. Repeating the amplification steps cyclically results in a large amount of product in a short time (10 – 90 minutes). The PCR products were sent to Eurofin MWG Operon (Norway) for analysis of the PCR sequence. These sequences were then compared to sequences in the BLAST nucleotide database and the ribosome database project to confirm that this was the ATCC9610 strain of *Y.enterocolitica.*

MALDI-TOF

Matrix Assisted Laser Desorption Ionisation time-of-flight (MALDI-TOF) mass spectrometry can be used to determine whole bacteria taxonomy (Lay, 2001). It is also used for the analysis of proteins, peptides, glycoproteins, oligosaccharides, and oligonucleotides. MALDI is based on the bombardment of sample molecules with a laser light (337nm) to bring about sample ionisation. The sample is mixed with a highly absorbent matrix, which transforms the laser energy into excitation energy for the sample. This leads to sputtering of analyte and matrix ions from the surface of the mixture (Ashcroft, 2011) and this is measured by the computer. Each bacteria strain has a unique mass spectra fingerprint, because of differences in protein amount and types of proteins present. The bacteria taxonomy can be identified by comparison to archived spectra (Holland et al., 1996).

One colony from a BHI plate with *Y.enterocolitica* was transferred to a chip. 1-2µL matrix (α-Cyano-4-hydroxycinnamic acid (CHCA)) was added on top of the *Y.enterocolitica* colony and then airdried before the chip was placed in the mass spectrometer (Bruker Daltonics, Germany). The mass-spectra achieved were compared to a database provided by Bruker Taxonomy (Bruker Daltonics, Germany).

2.3 Isolation of Granulocytes

Human venous blood (30-60mL) was obtained from healthy female volunteers (ages 23-50) in EDTA vacuettes. Neutrophil granulocytes were separated from EDTA blood by dextran sedimentation followed by a standard density gradient centrifugation as previously described by Bøyum (1991). 30 mL EDTA blood from individual donors was mixed with 3 mL 6% dextran (in 0.9% NaCl) and left for sedimentation at room temperature for 30 minutes. The supernantant containing granulocytes was subjected to Lymphoprep density gradient centrifugation at 690**g* for 15 minutes. The pellet was washed in 10mL 0.9% NaCl and then resuspended in 5mL 0.83% NH_4Cl for 7 minutes to lyse the erythrocytes, and then centrifuged for 7 minutes (690**g*). This process was repeated until the pellet was white. The cells were resuspended in HEPES-buffered (20mM) HBSS ($CaCl_2$ 1.3 mM, KCl 5.4 mM, KH_2PO_4 0.44 mM, $MgCl_2$ 0.49 mM, $MgSO_4$ 0.41 mM, NaCl 140 mM, $NaHCO_3$ 4.2 mM, Na_2HPO_4 2.7 mM), pH 7.4 with 5 mM glucose. The number of granulocytes was determined with ADVIA60 hematology system. The cells were kept on ice (approximately 4ºC) until used (maximum 2 hours).

2.3.1 Chemiluminescence Assay

The luminol-amplified chemiluminescence was measured by the luminometer FLUOStar Optima (BMG LabTech, Germany). The reaction mixture (250µL) contained 0.1 mM Luminol, $2x10^5$ cells and increasing concentrations of the test compounds as seen in section I.iii in the appendix. Chemiluminescence was measured for approximately 60 minutes at 35ºC. Each concentration was tested in triplicate and the experiments were repeated at least three times. The cells and reagents were prepared in HEPES-buffered (20mM) HBSS with 5 mM glucose. A positive control with PMA ($1*10^{-8}$M) was included in all experiments. This is a known Protein Kinase C (PKC) activator, and activates the NADPH oxidase system in granulocytes, which leads to ROS production (Benna et al., 1997; Hsu et al., 2008).

2.3.2 Calculation of Results

The production of ROS in granulocytes exposed to bacteria and toxicants was compared with a DMSO-control. A DMSO effect was calculated based on all the experiments. The effect was 90% ± 20% of luminol with HBSS only, which was not significant.

The chemoluminescence produced in the experimental assays was given as the area under curve (AUC). AUC varied substantially between the experiments, even for the controls (HBSS and DMSO), which made it difficult to compare results. To compare all the experiments in a more convenient way, the results from each experiment were transformed to multiples of the control (DMSO). All the results are therefore presented as multiples of DMSO's AUC.

The mixtures of environmental toxicants were made at a ratio according to the EC_{50} ratio of the toxicants. EC_{50} was decided from the asymmetrical Hill-curve for each of the environmental toxicants (formula 5) with Graph Pad Prism (GraphPad Software, Inc., USA). These curves were also the basis for the Bliss and Loewe predictions of the mixtures.

$$\text{Effect} = \text{Min} + \frac{(\text{Max} - \text{Min})}{\left[1 + \left(\frac{c}{\text{EC}_{50}}\right)^{-p}\right]},$$

Formula 5 The asymmetrical Hill shape function. Min = minimal observed effect; Max = maximal observed effect; *c* = concentration of test agent; EC_{50} = the concentration of test agent yielding half-maximal effects and *p* is the slope parameter (Rajapakse et al., 2002).

2.3.3 The Bliss Model

The Bliss independence model was calculated for all the mixtures. The Hill-shape curve function of each chemical in the mixture was used as a basis for the Bliss prediction. A standard deviation (SD) was calculated based on the individual Hill-shape curve from the 3-4 experiments with each chemical in the mixture. This SD was transformed the same way as the Bliss predictions. This was done to show that individual variance strongly affected the predictions.

For this model to work, the effect of each substance needs to be a number between zero and one. To manage this, all effects were divided by the maximum effect (the top of the Hill-

shape curve) of the substance in the mixture with the highest effect. It was necessary to perform this transformation on the observed values as well, to be able to compare the values with the predictions.

2.3.4 The Loewe Model

The Loewe additivity model was calculated for mixtures with a positive Hill slope. Bisphenol A, which had a negative hill slope, clearly reacted in a different manner than the rest, and the Loewe additivity model was not applicable. The original approach from Loewe and Muischnek (1926) did not include a way to calculate the SD. To check if the eventual deviation from the model was due to chance or of actual relevance, a standard deviation was calculated based on the individual Hill-shape curve from the experiments with each chemical.

To calculate the Loewe effect, formula (3), was used to calculate EC_{mix}, which is the effective concentration of the mixture. Then the effect of the mix was found in a table with theoretical EC_{mix} vs. effect. The theoretical EC_{mix} is based on what dose of each of the components gives the same effect according to a Hill-shape curve calculated from experiments with the single compounds. The slope of the individual dose-response curves differs between the individual constituents of the mixtures, but according to Berenbaum (1989) this model is independent of the shape of the individual dose-response curves.

The Loewe model was used on effects between zero and one. To accomplish this, new hill-shape curves were calculated. The maximum (from the hill-shape-formula) of the compound with the highest maximum in the binary mixture were used, and relative dose-response curves were calculated. If the overall highest result had been used, it would not have been possible to calculate Hill-shape curves for some of the compounds.

2.4 Stock Solutions

All stock solutions were dissolved in DMSO, except TBBPA which was dissolved in methanol, and LPS and Pam3CSK4 which were dissolved in 0.9% NaCl. In all experiments the concentration of solvent never exceeded 0.5%, with one exception. 500µL stock solution with *Y.enterocolitica* was mixed with 9.5 mL human neutrophil granulocytes in HBSS, which caused a five percent dilution of the cell-solution. When mixing this with toxicants and luminol the total solution was diluted two percent with PBS. This was necessary to inhibit

growth in the stock solution of bacteria during the experiment, so that all the parallels got the exact same amount of bacteria.

2.5 Statistics

The data are given as mean±SD. Statistical analysis was performed with Graph Pad Prism. Data were considered significant at a level of $P < 0.05$. A Kolmogorov-Smirnov test indicated that the responses fit a Gaussian distribution. Analysis of variance (ANOVA) was used to calculate significance for all the dose-response curves and the Dunnet Multiple Comparison Test was used as a post-test to determine significances for each concentration compared to the control. A two-way ANOVA was used to calculate significance between treatments (LPS/bacteria/time) with different concentrations of toxicants or bacteria present. A Bonferroni post-test was used to indicate significances for each concentration compared to the control. A two way ANOVA investigates; if the dose has the same effect at all values of treatment (LPS, bacteria, time); if the dose affects the result; and if the treatment affect the result (Motulsky, 2003). Below all the figures where a two-way ANOVA has been used, a parenthesis with the result will be presented (interaction; dose; treatment;).

3 Results

3.1 Whole Blood Model

3.1.1 Whole Blood Experiments with Bacteria

The viability of the bacteria in whole blood was tested by comparing growth in different ratios of PBS. CFU was counted at t=0 (Fig. 12), and after six and 24 hours, to see if the bacteria were still viable. The viability of the bacteria in blood varied between subjects (data not shown) which may cause variations in the results. As seen in figure 12, the actual amount of CFU calculated did not predict CFU closely (see appendix II.i). Because of these variations, later experiments with granulocytes were done with the same stock of bacteria during the same day and CFU was counted to confirm the bacteria amount used in the experiment.

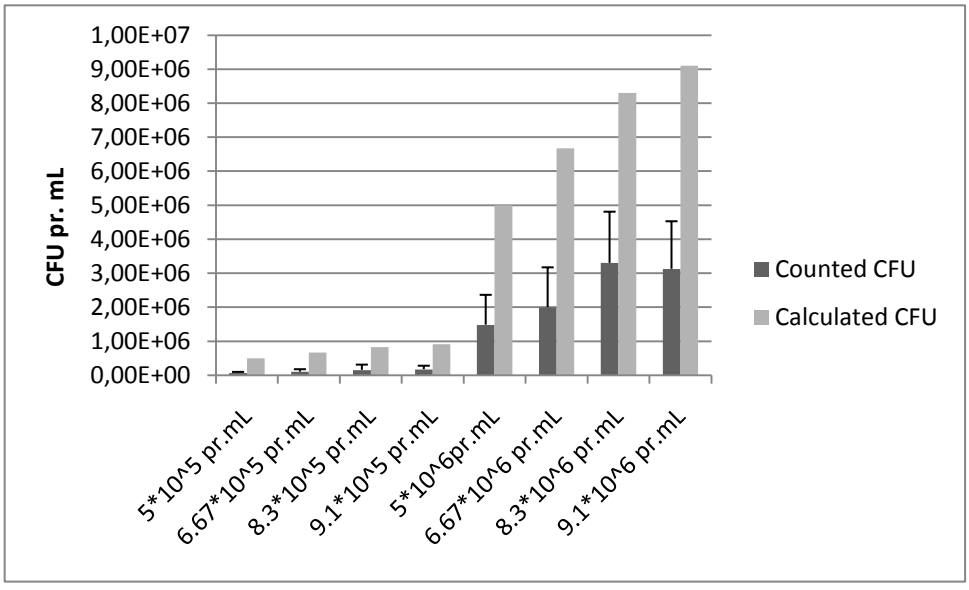


Figure 12 Comparison of counted CFU on BHI plates (t=0) with calculated CFU by formula (4) (n=3).

BHI-medium caused some background in cytokine production (data not shown (n=4)). This problem was reduced by washing and dissolving the bacteria in PBS (Fig. 13). Increasing the amount of bacteria induced increased production of Tnf-α (Fig. 13). This response was somewhat suppressed after 24 hours, not significantly. The cytokine production after exposure to *Y.enterocolitica* varied more between subjects compared to the LPS response.

This is probably caused by the difference in amount of bacteria present. When we calculated Tnf-α production per CFU present it seemed that the amount of PBS had no influence on Tnf-α production (Fig. 14).

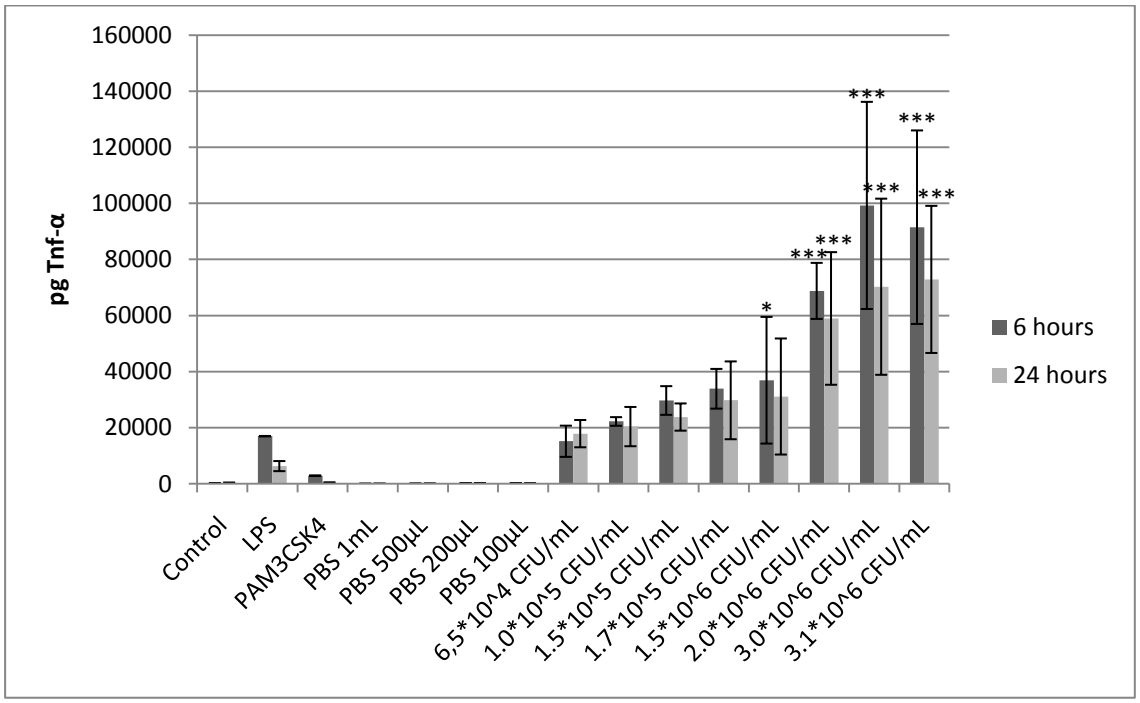


Figure 13 Tnf-α –production after exposure to different concentrations of *Y.enterocolitica* with different concentrations of PBS in 1mL whole blood for 6 and 24 hours. Two-way ANOVA with Bonferroni posttest indicated statistical significance between the controls and different concentrations of *Y.enterocolitica*. (interaction: ns, CFU: *, time: ns) ***p<0.001 **p<0.01 and *p<0.05 (n=3)**

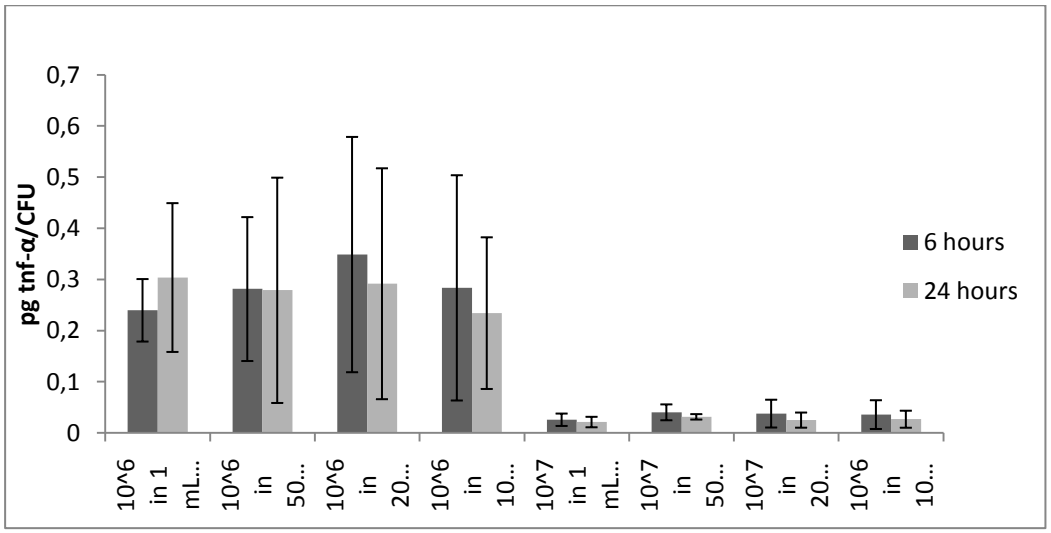


Figure 14 Tnf-α production pr. CFU. Two-way ANOVA indicated that only CFU accounts for the variance (interaction: ns, CFU: *, PBS: ns) ***p<0.001 (n=3)**

The morphology of the bacteria changed over time and because of this it was necessary to confirm the bacteria species. This was done in approximately 50% of the experiments with

Y.enterocolitica, of which one colony was checked with PCR or MALDI-TOF. The results were always positive for the *Y.enterocolitica* serotype used in these experiments (see appendix II.ii).

3.1.2 Whole Blood Experiments with Environmental Toxicants

Whole blood was exposed to environmental toxicants for six and 24 hours to investigate induction of cytokine production. Eight different chemicals were screened at high concentrations. Apparently, all the test substances induced a small increase in Tnf-α production in some of the donors, but because of the huge variation between donors the results were not significant (Fig. 15).

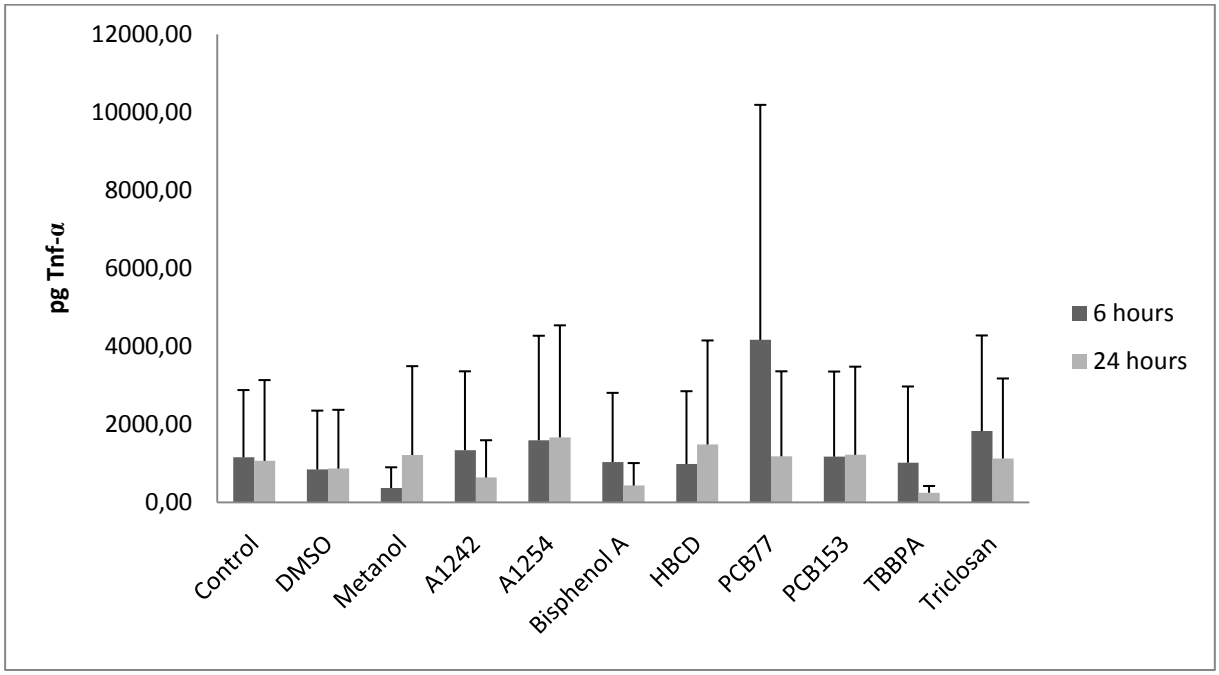


Figure 155 Tnf-α production in whole blood after exposure to different environmental toxicants at 50µM. PCB77 is at 10 µM. The positive control LPS are at 14000 pg/mL at 6 hours (data not shown). Two-way ANOVA indicated no statistical significance between the controls (DMSO/Methanol) and the environmental toxicants. (n=5)

A dose-response relationship was then evaluated for three of the chemicals, namely PCB77, A1254 and HBCD. However, in these experiments there was no trend in formation of Tnf-α and no significant formation of IL-1β (Fig 16 -17).

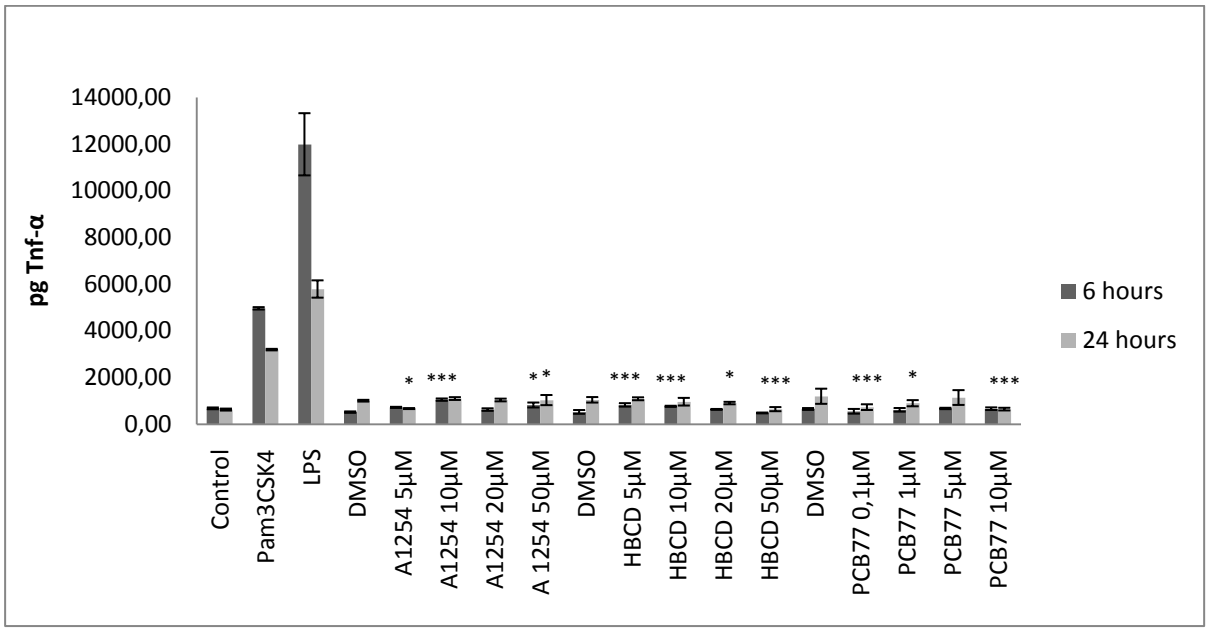


Figure 16 Tnf-α production in whole blood after exposure to different environmental toxicants at different concentrations after 6 and 24 hours incubation. Two-way ANOVA with Bonferroni posttest indicated statistical significance between DMSO and the environmental toxicants, there were no relationship between increasing/decreasing dose and response making the result difficult to interpret. (A1254: interaction: *, dose: * , time:***; HBCD: interaction:***, dose:***, time:***; and PCB77: interaction:**, dose:***, time:***) ***p<0.001 **p<0.01 and *p<0.05 (n=5)**

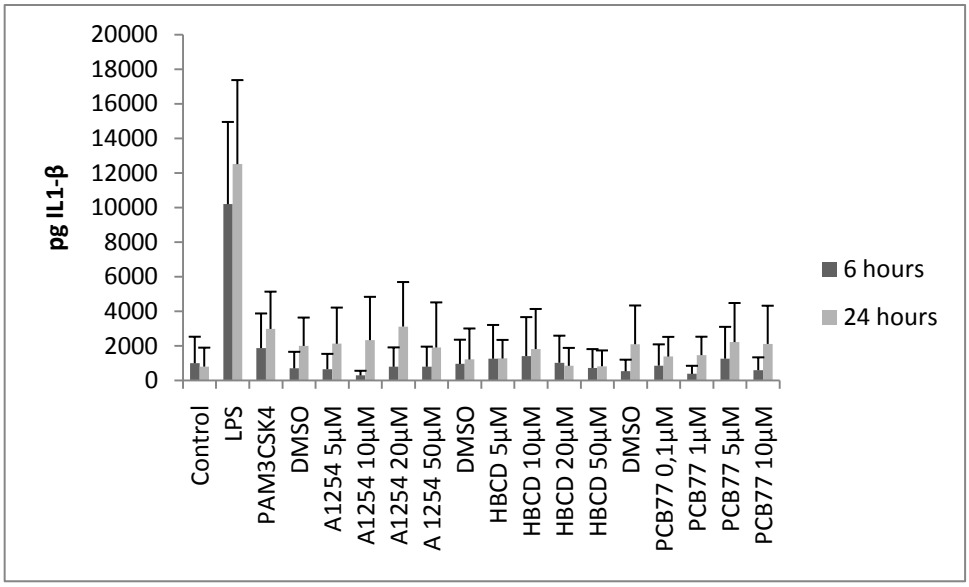


Figure 17 IL1-β production in whole blood after exposure to different environmental toxicants at different concentrations after 6 and 24 hours incubation. Two-way ANOVA with Bonferroni posttest indicated no statistical significance. (n=3)

A dose-response experiment with LPS and Pam3CSK4 was performed to determine which concentrations to be used when exposing whole blood to mixtures of bacterial analogs and environmental toxicants. We wanted a concentration in the middle of the linear slope, so that the environmental toxicants could show suppressing or activating effects on the expected

effect of the bacterial analogs. Both LPS and Pam3CSK4 induced a concentration dependent increase in Tnf-α production (Fig.18-19). In the mixture experiments, 1 ng/mL was chosen for LPS and 100ng/mL for Pam3CSK4. Time seemed to have a suppressing effect on the induction of Tnf-α by LPS and Pam3SCK4. Two chemicals, namely A1254 and PCB77 were chosen to be exposed together with LPS and Pam3CSK4. The environmental toxicant had no significant effect on Tnf-α formation induced by LPS and PAM3CSK4 (Fig. 20-21).

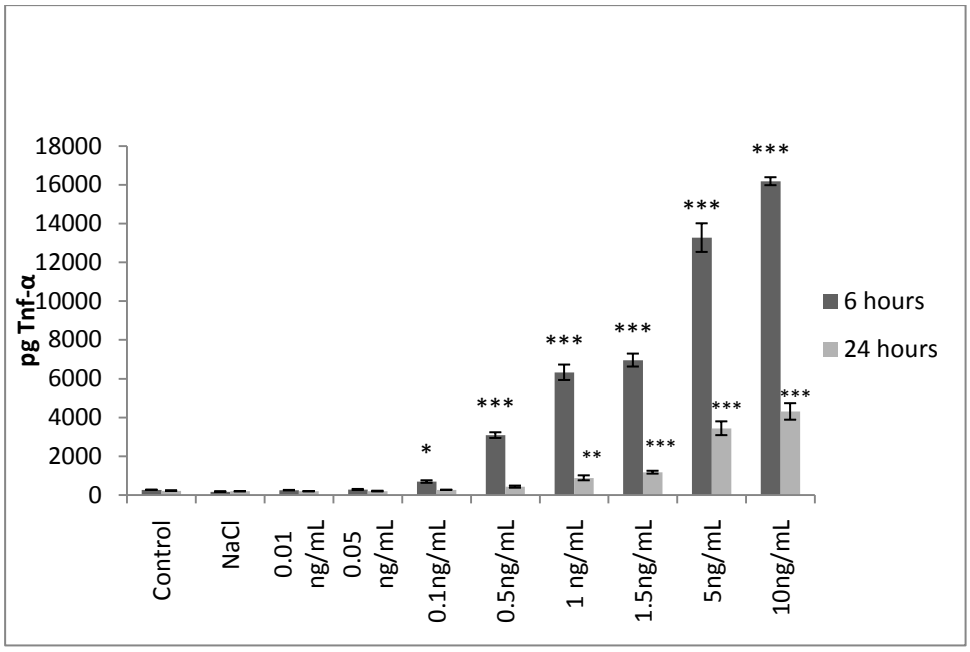


Figure 18 Tnf-α production in whole blood after exposure to 0.01 – 10ng/mL LPS. (n=3) Two-way ANOVA with Bonferroni posttest indicated statistical significance between NaCl and LPS. (interaction:*, dose:***, time:***) ***p<0.001 **p<0.01 and *p<0.05 (n=3)**

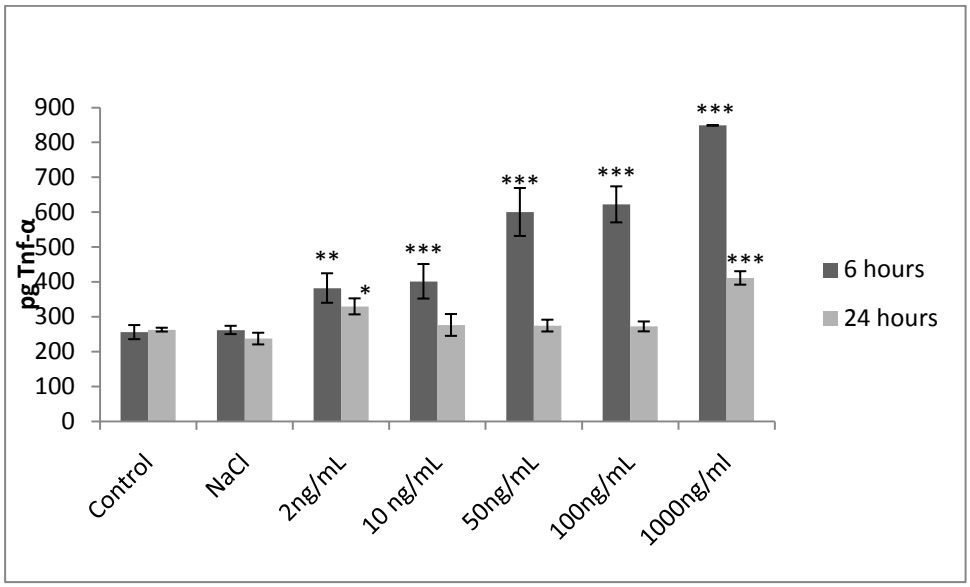


Figure 19 Tnf-α production in whole blood after exposure to 2 – 1000ng/mL Pam3CSK4. Two-way ANOVA with Bonferroni posttest indicated statistical significance between NaCl and Pam3CSK4. (interaction:*, dose:***, time: ***) ***p<0.001 **p<0.01 and *p<0.05 (n=3)**

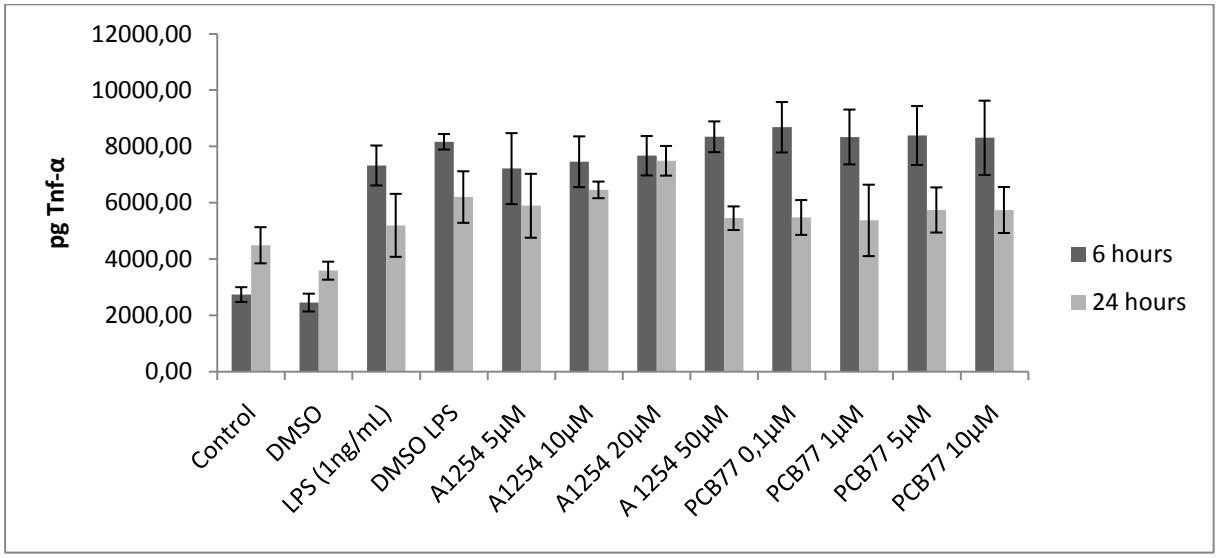


Figure 20 Tnf-α production in whole blood after exposure to A1254 and PCB77 at different concentrations with 1ng/mL LPS. Two-way ANOVA with Bonferroni posttest indicated statistical significance between DMSO+LPS and A1254 or PCB77. (A1254: interaction: **, dose: ns, time: * PCB77: interaction: ns, dose: ns, time:***) ***p<0.001 **p<0.01 and *p<0.05 (n=5)**

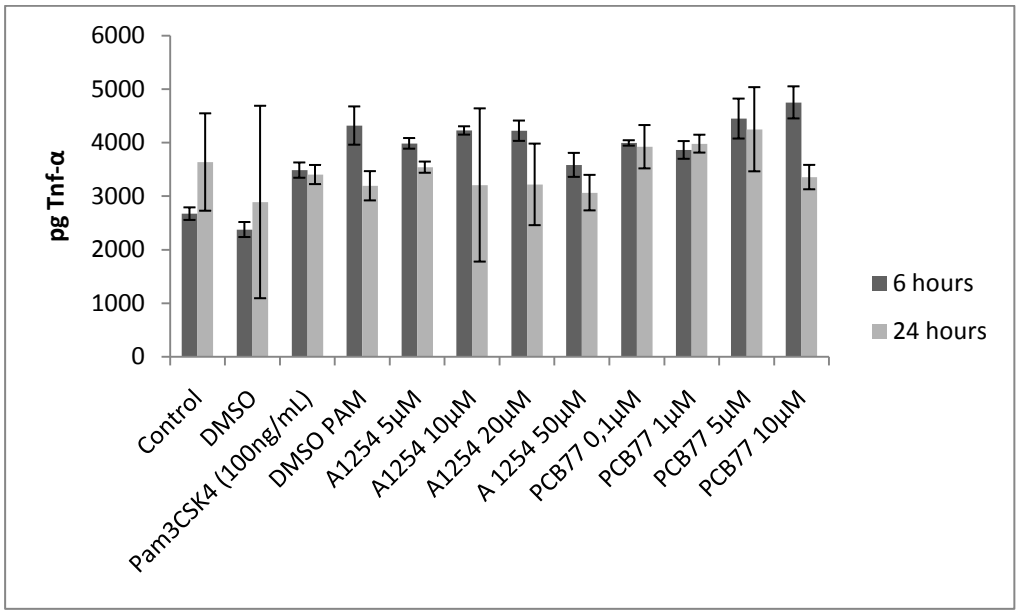


Figure 21 Tnf-α production in whole blood after exposure to A1254 and PCB77 at different concentrations with 100ng/mL PAM3CSK4. Two-way ANOVA with Bonferroni posttest indicated statistical significance between DMSO+Pam3CSK4 and A1254 or PCB77. (A1254: interaction: ns, dose: ns, time: * PCB77: interaction:***, dose:*, time:***) ***p<0.001 **p<0.01 and *p<0.05 (n=5)**

3.2 ROS Production in Granulocytes

3.2.1 Dose Response with Environmental Toxicants

Isolated granulocytes were exposed to different concentrations of five environmental toxicants to see if they induced ROS production. All the environmental toxicants tested showed a dose-response relationship, as pictured in figure 22 and 23, with EC_{50} values as shown in table 4. There was considerable variation in response between the donors, figure 22 and 23.

A1254 had a lower induction of ROS than ortho-chlorinated PCB153. Bisphenol A surprisingly induced a negative dose-response relationship. Triclosan and HBCD had a positive but small dose-response effect on ROS production.

Table 4 EC_{50} of raw data.

	EC_{50}
A1254 (µM)	7.811
Bisphenol A (µM)	1.516
HBCD (µM)	15.98
PCB153(µM)	8.431
PMA (nM)	4.391
Triclosan (µM)	6.245

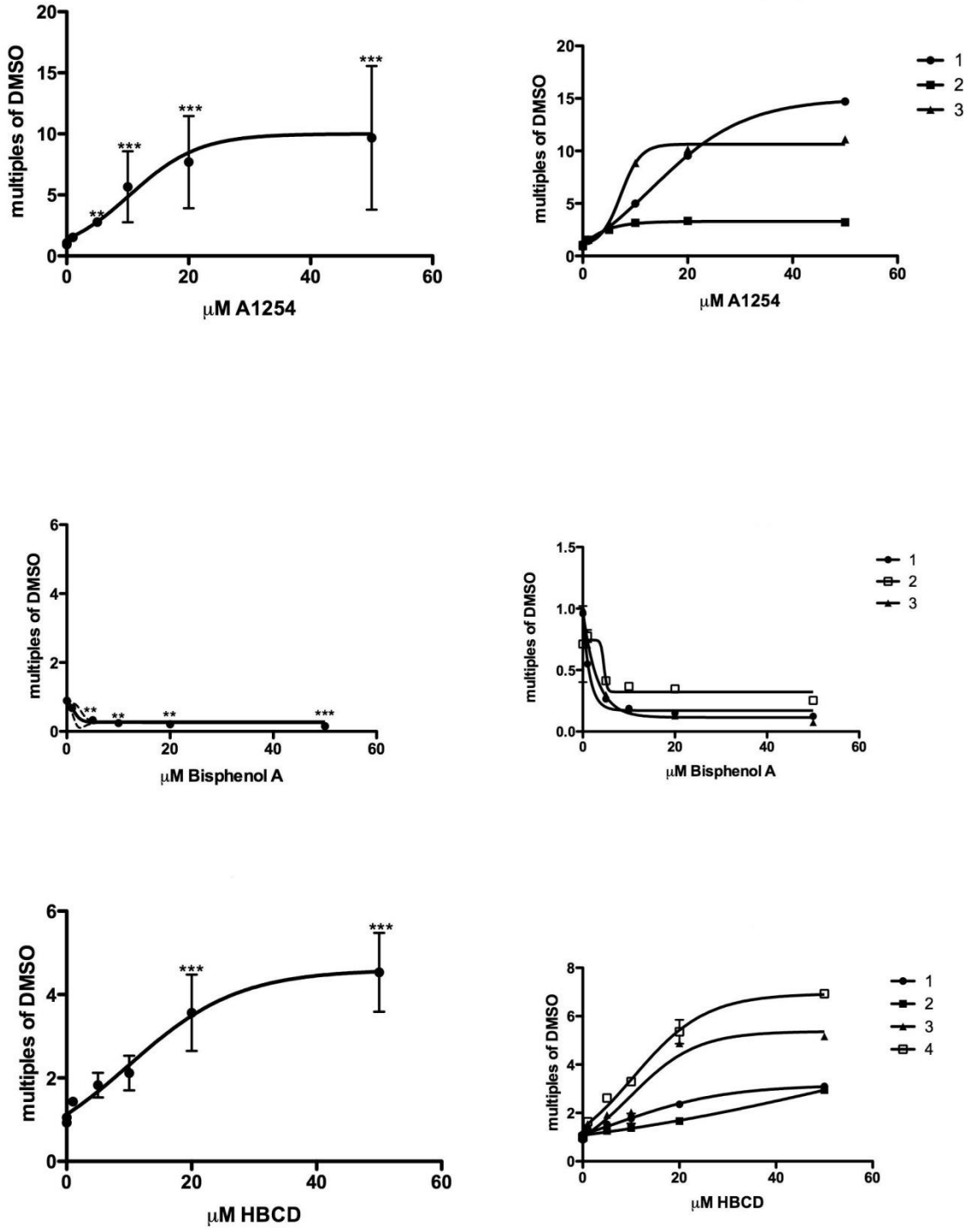


Figure 22 The dose-response relationship for A1254, Bisphenol A and HBCD. The results from each separate experiment are shown to the right and the total result with indications of significance is shown to the left. One-way ANOVA followed by Dunnet's multiple Comparison test indicated statistically significant differences between exposure groups with DMSO as control. *** p<0.001, **p<0.01 and *p<0.05 (n=3-4)

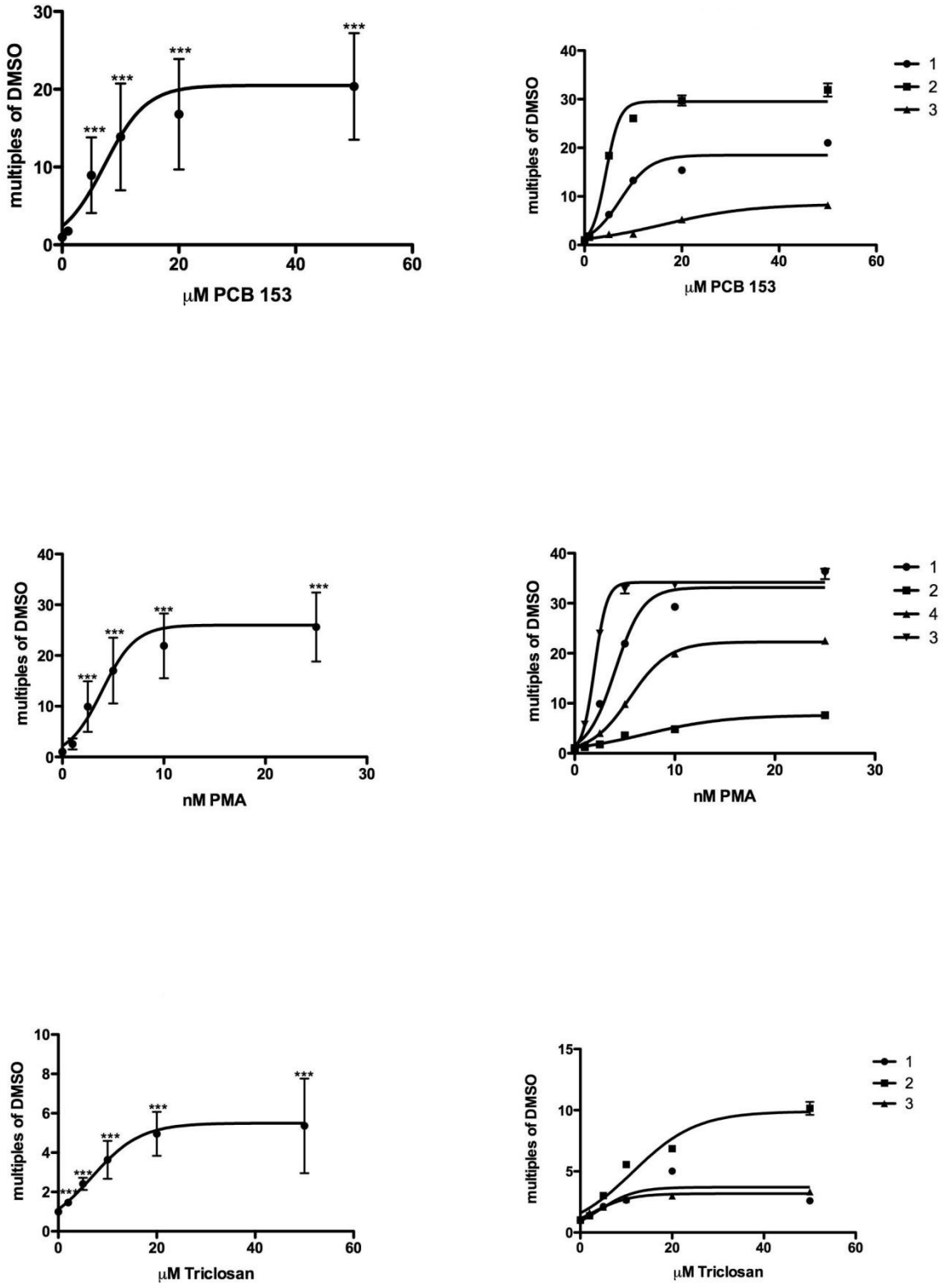


Figure 23 The dose-response relationship for PCB153, PMA and Triclosan. The results from each separate experiment are shown to the right and the total result with indications of significance is shown to the left. One-way ANOVA followed by Dunnet's multiple Comparison test indicated statistically significant differences between exposure groups with DMSO as control. *** p<0.001, **p<0.01 and *p<0.05 (n=3-4)

The ROS production was initiated at different times for the different substances, illustrating different kinetics of the ROS formation (Fig. 24). Triclosan was early up and early down, while A1254, HBCD, PCB153 and PMA started at approximately the same time but with different shapes or potency of the reaction.

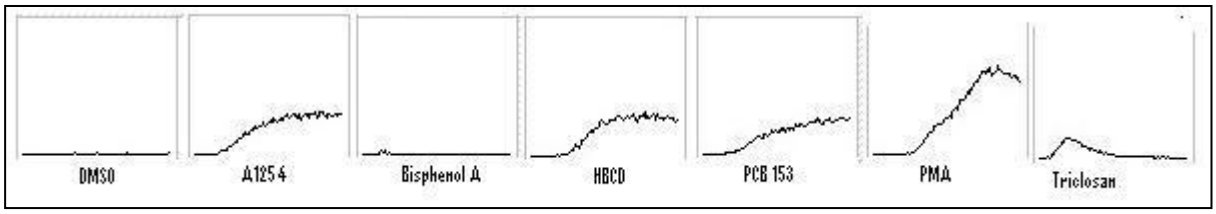
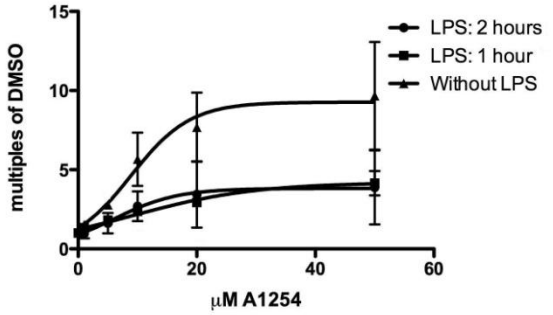
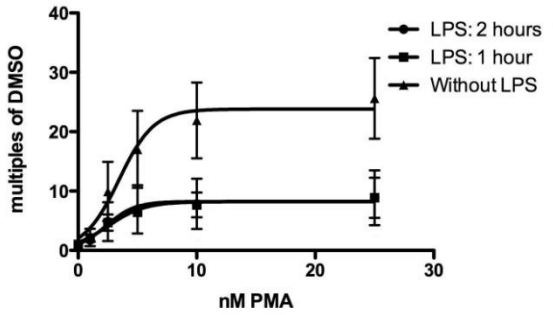


Figure 24 Luminescence measurements during 1 hour after exposure. (Pictures shows the graph for the maximum concentration of each of the test compounds, except for PMA which is shown at 10nM)

3.2.2 LPS Priming of Granulocytes

Human neutrophil granulocytes were primed with 1ng/mL LPS for 2 hours on ice or for 1 hour at room temperature and were then exposed to five concentrations of PMA, A1254 or Triclosan. The priming seemed to suppress the response of the toxicants investigated (Fig.25).





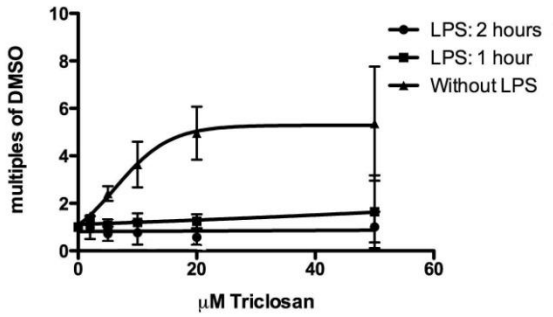


Figure 25 The original Dose-Reponse curves from figure 22 and 23 for A1254, PMA and Triclosan together with the response from cells primed with 1ng/mL LPS exposed to A1254, PMA and Triclosan. LPS did not give a response alone at these concentrations (data not shown). Two-way ANOVA indicated statistical significance between groups, see table 5. *** p<0.001, **p<0.01 and *p<0.05 (n=3)

Table 5 Statistical significance of treatment with LPS

	A1254		PMA		Triclosan	
	Two hours	One hour	Two hours	One hour	Two hours	One hour
Interaction	***	**	**	*	***	**
LPS treatment/Dose	***/***	***/***	***/***	***/***	***/***	***/***

3.2.3 *Y.enterocolitca* with Granulocytes

To find an approximate concentration where *Y.enterocolitica* started to affect granulocyte ROS production, different amounts of CFU (50-250 000) were investigated (Fig. 26). The goal was to decide on a concentration of *Y.enterocolitica* to use in combination with environmental toxicants on neutrophil granulocytes. *Y.enterocolitica* gave a concentration-dependent increase in ROS formation. 250 000 CFU *Y.enterocolitica* were chosen, which gave a response of 2.485 ±0.533 multiples of HBSS.

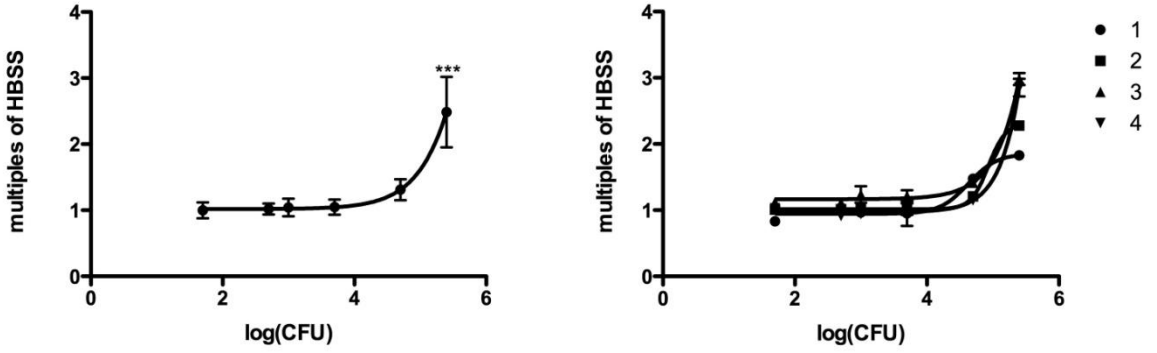


Figure 26 The granulocytes were exposed to 50-250 000 CFU *Y.enterocolitica*. One-way ANOVA followed by Dunnet's multiple Comparison test indicated statistically significant differences between exposure groups with HBSS as control. *** p<0.001, **p<0.01 and *p<0.05 (n=4)

Since HBSS contains glucose it was likely that *Y.enterocolitica* replicated before and during the experiments. Growth depends on a number of factors, such as temperature, medium and nourishment. The growth of *Y.enterocolitica* in HBSS has not been investigated, but counting CFU on agar showed $46.6*10^6$ CFU in the original stock diluted in PBS (t=0); in HBSS the stock grew to $66.3*10^6$ CFU in HBSS within 1 hour.

In the experiments with environmental toxicants mixed with *Y.enterocolitica* a stock solution of approximately $50*10^6$ CFU ($54.6*10^6$ CFU) was made with PBS instead of HBSS, to inhibit growth. 500µL of this solution was mixed with the cells just before the experiment started. The environmental toxicants chosen to investigate mixture effects with *Y.enterocolitica* were A1254, PCB153 and Triclosan.

As seen in figure 25, co-exposure with *Y.enterocolitica* did not change the results for A1254. It seems that the effect of PCB153 together with *Y.enterocolitica* was affected more strongly

(Fig. 27-28). Triclosan showed a bell-shape curve (Fig. 29), with somewhat reduced effects at high concentrations of Triclosan.

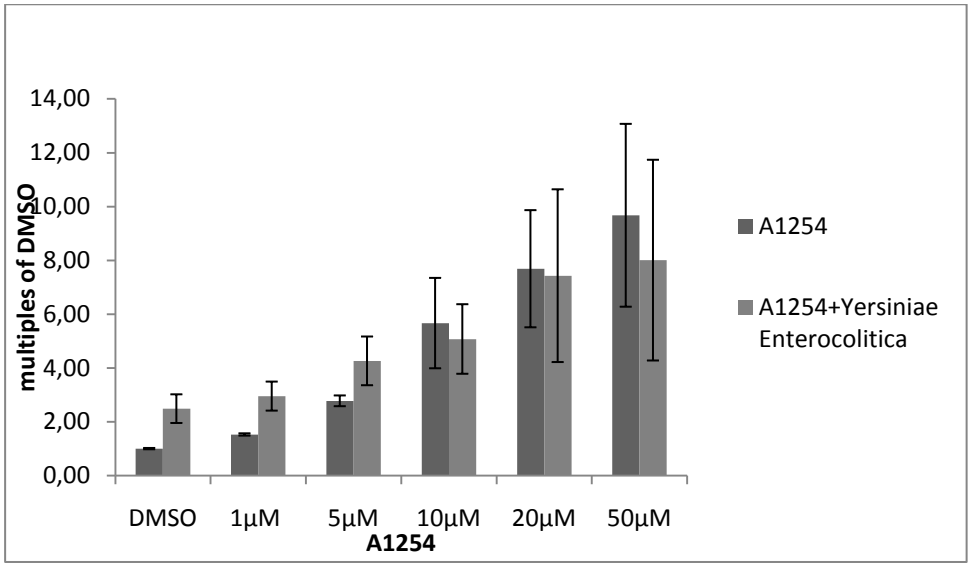


Figure 27 The results of A1254+*Y.enterocolitica* calculated as the result of multiples of DMSO+ *Y.enterocolitica* and transformed with +1.485 ±0.533 to take into account the effect of 250 000 CFU *Y.enterocolitica* alone. Two-way ANOVA showed no significant differences after treatment with *Y.enterocolitica.* (interaction: ns, dose: *, *Y.enterocolitica*: ns) *** p<0.001, **p<0.01 and *p<0.05 (n=3)**

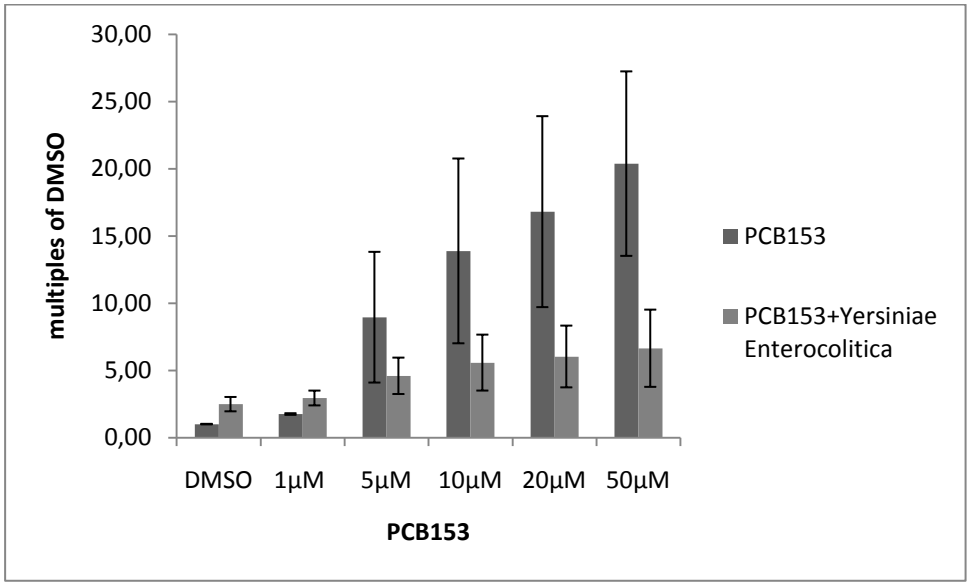


Figure 28 The results of PCB153+*Y.enterocolitica* calculated as the result of multiples of DMSO+*Y.enterocolitica* and transformed with +1.485 ±0.533 to take into account the effect of 250 000 CFU *Y.enterocolitica* alone. Two-way ANOVA showed significant differences after treatment with *Y.enterocolitica.* (interaction: *, dose: *, *Y.enterocolitica*: ***) *** p<0.001, **p<0.01 and *p<0.05 (n=3)**

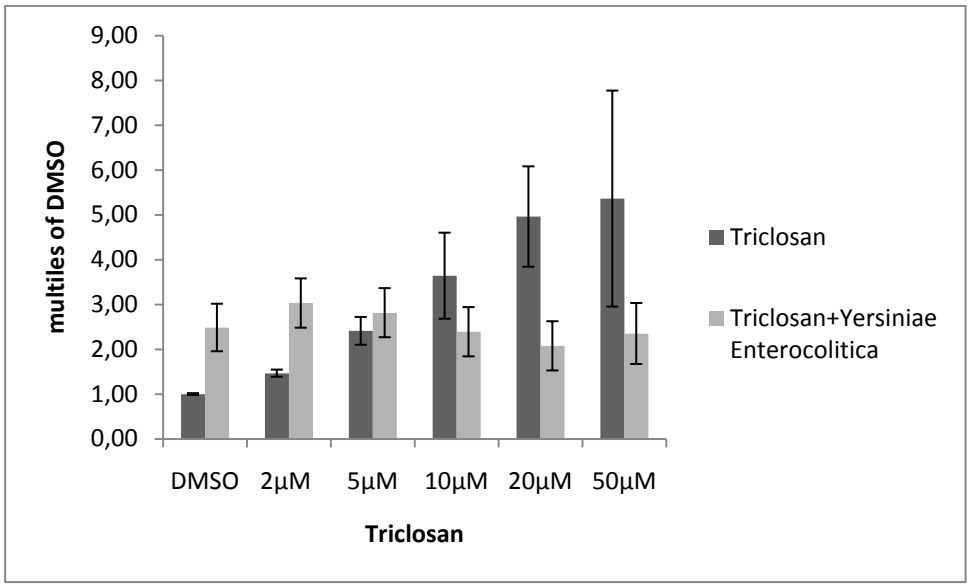


Figure 29 The results of Triclosan+*Y.enterocolitica* calculated as the result of multiples of DMSO+ *Y.enterocolitica* and transformed with +1.485 ±0.533 to take into account the effect of 250 000 CFU *Y.enterocolitica* alone. Two-way ANOVA showed significant differences after treatment with *Y.enterocolitica.* (interaction: *, dose: **, *Y.enterocolitica*: ns) *** p<0.001, **p<0.01 and *p<0.05 (n=3)**

3.2.4 Mixture Effects of Environmental Toxicants.

Binary mixtures of toxicants were made (table 5). Bisphenol A, Triclosan and HBCD were mixed with A1254, PCB153 and PMA. The mix was made in a ratio according to their EC_{50} values (table 4 and 6). Then neutrophil granulocytes were exposed to five or six different concentrations of these mixtures.

Table 6 The ratio between the constituents of the mixtures.

Mixtures	Ratio
Triclosan/A1254	1.2
Triclosan/PCB153	1.4
Triclosan/PMA	1427
HBCD/Triclosan	2.5
Bisphenol A/A1254	5.2
Bisphenol A/PCB153	5.6
Bisphenol A/PMA	343
HBCD/A1254	2
HBCD/PCB153	3.8

The binary mixtures induced an apparent dose-dependent increase in ROS formation, with the exception of three mixtures with Triclosan (Fig. 30). Due to large individual variations in ROS response, only Triclosan mixed with A1254 and Triclosan mixed with HBCD had reactions significantly different from the control. Three of the mixtures with Triclosan seemed to have a reduced formation of ROS at higher concentrations.

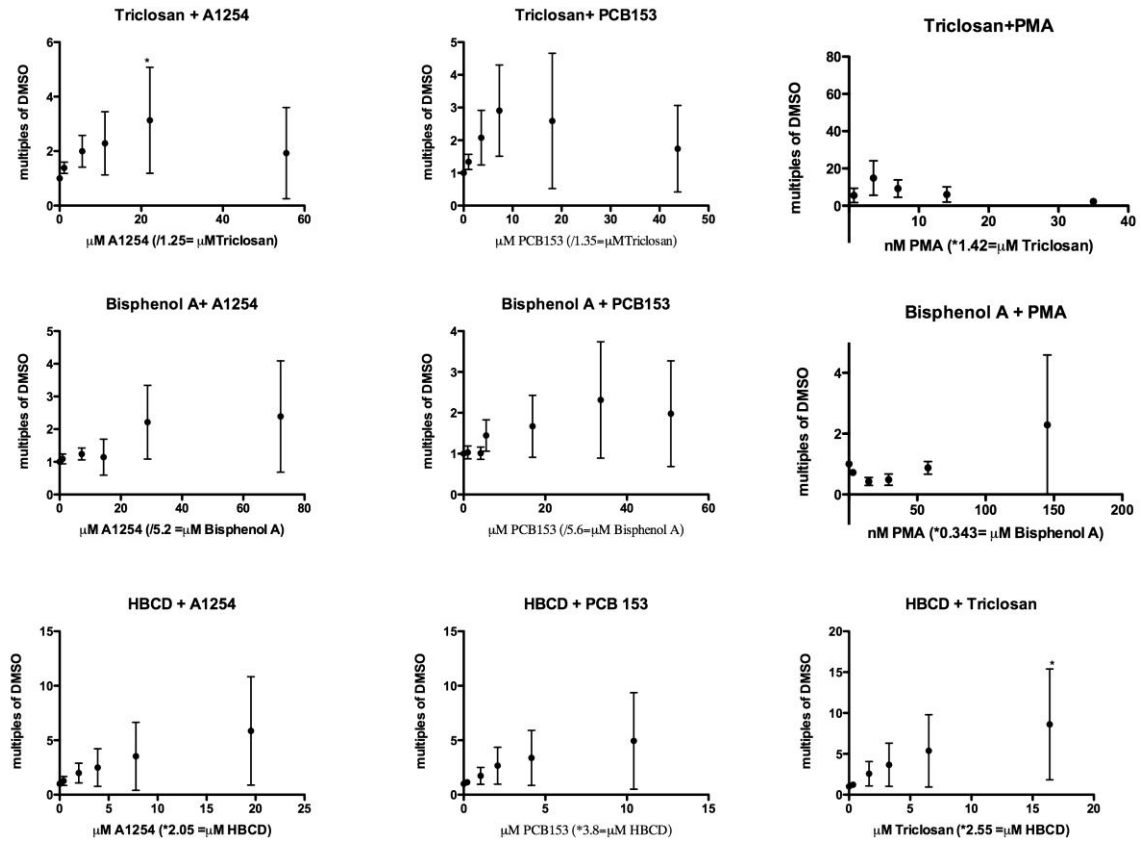


Figure 30 Binary mixture effects. Information in parantheses states whether to multiply (*) or divide (/) by X to learn the concentration of the other constituent of the mixture. Only Triclosan+A1254 and Triclosan+HBCD have a result significantly higher than the controls. One-way ANOVA followed by Dunnet's multiple Comparison test indicated statistical significant differences between exposure groups with DMSO as control. *p<0.05 (n=4)

It is interesting to note how the kinetics, as observed with AUC, changed for the mixtures (Fig. 31 and 32) compared to the kinetics of the single toxicants (Fig. 24). The kinetics even varied at different concentrations of the binary mixture of Triclosan and PMA (bottom of Fig 32). The mixtures with HBCD or Bisphenol A displayed kinetics similar to a combination of the kinetics of the single constituents (Fig. 31-32).

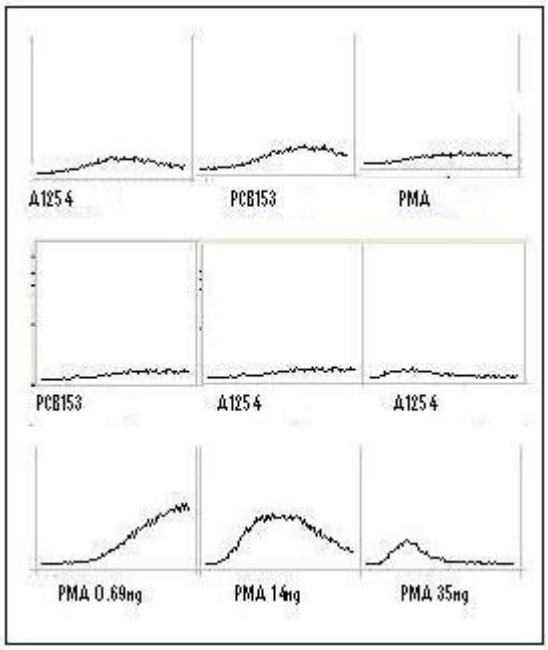


Figure 32 AUC for Bisphenol A mixtures at the maximum concentration used for each of the compounds(top) and Triclosan mixtures (middle and bottom) with concentrations shown for mixture with PMA. PCB153 at 18.1µM and A1254 at 5.5µM and 55µM

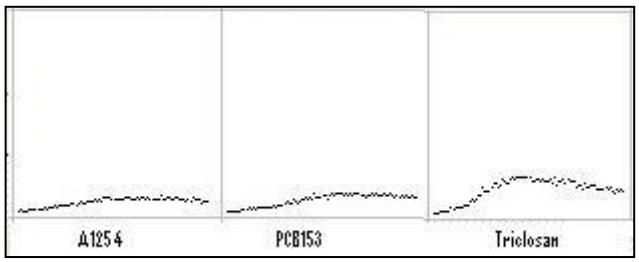


Figure 31 AUC for HBCD mixtures at the maximum concentration used for each of the compounds.

To compare the observed ROS-formation of the mixtures with the values predicted by the Loewe or the Bliss model, each of the values were presented in a graph. All results were transformed as described in sections 2.3.2 - 2.3.4. Normally an isobol diagram would have been designed to investigate if a mixture was additive or not (appendix I.v). This was not possible with these mixtures because of the variation between donors and the shape of the dose response curves. Graph Pad Prism was used to perform multiple regression analysis. The results were given in the two-way ANOVA format. If a prediction holds, there should be no significant difference between the curve of the observed values and the curve of the predicted values.

Mixtures with Triclosan

Mixtures with Triclosan and A1254, PCB153 and PMA did not follow predictions closely with a predominant antagonistic trend (Fig. 33, 35-36). The binary mixture with HBCD and Triclosan reacted differently than the other mixtures with Triclosan (Fig.34). Here, the mean ROS production was above the predicted values, but the differences were not significant due to the large variation between donors.

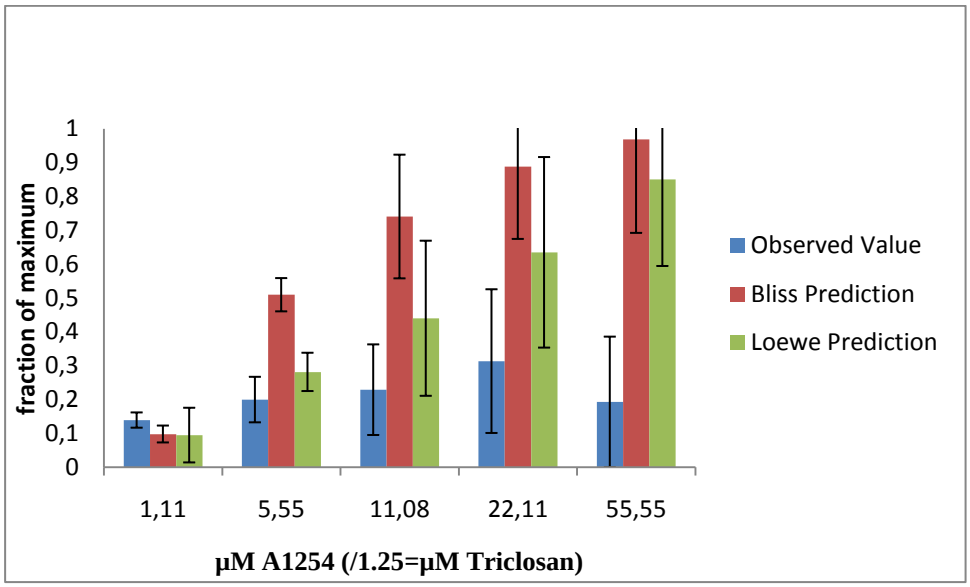


Figure 33 The experimental value and the predicted values for different concentrations of an EC_{50} ratio mixture of A1254 and Triclosan. Loewe Prediction vs. Observed Value: **p<0.01

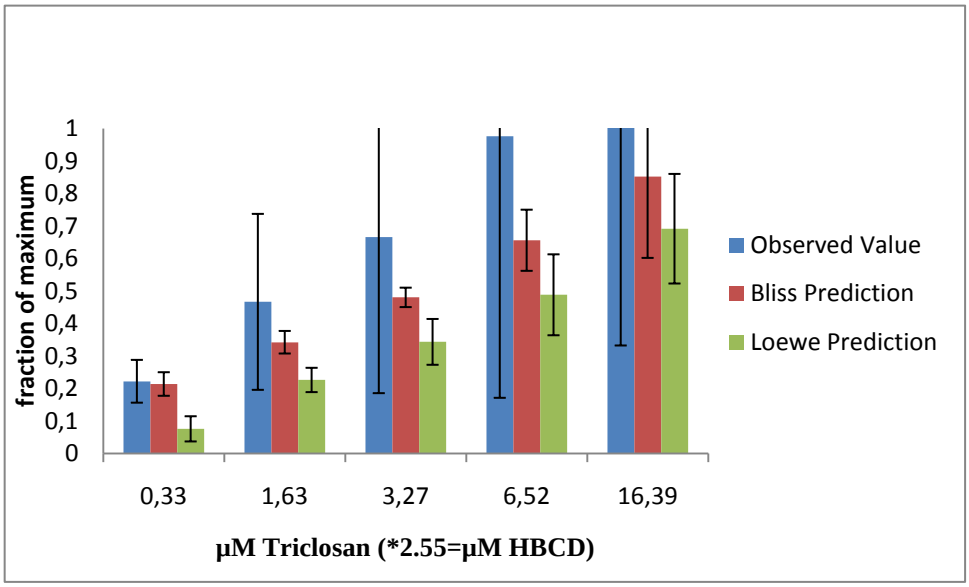


Figure 34 The experimental value and the predicted values for different concentrations of an EC_{50} ratio mixture of HBCD and Triclosan. No significant differences.

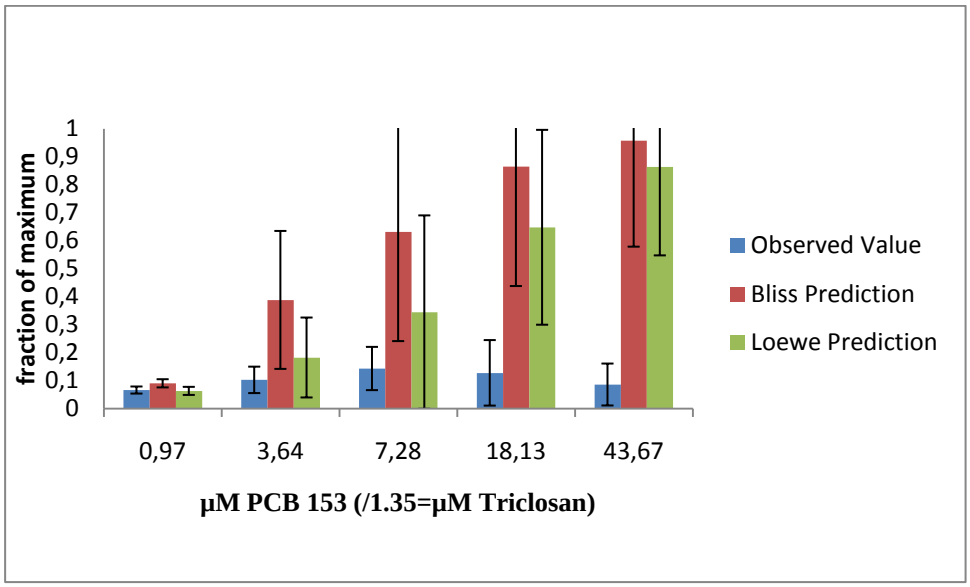


Figure 35 The experimental value and the predicted values for different concentrations of an EC_{50} ratio mixture of PCB153 and Triclosan. Loewe Prediction vs. Observed Value: ***p<0.001

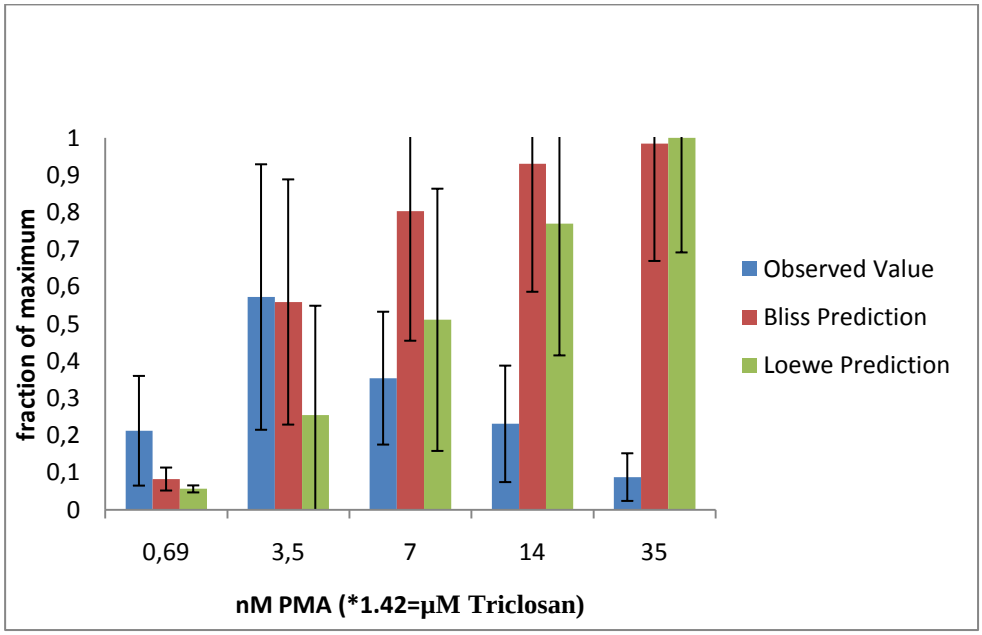


Figure 36 The experimental value and the predicted values for different concentrations of an EC_{50} ratio mixture of PMA and Triclosan. Loewe Prediction vs. Observed Value:*p<0.05

Mixtures with HBCD

The binary mixtures with HBCD and PCBs seemed to follow closely the Loewe prediction at most concentrations, whereas Bliss predicted higher effects than observed. Note, however, that the observed effects were not significantly different from the Bliss predictions (Fig. 37-38). In the mixture with PCB153 there was a trend toward antagonistic effects at higher concentrations compared to the predictions.

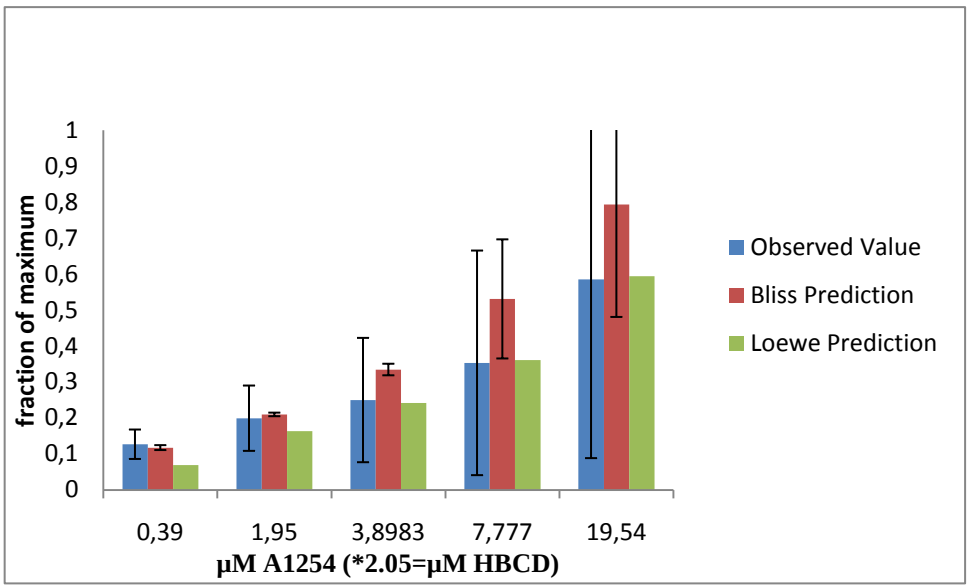


Figure 37 The experimental value and the predicted values for different concentrations of an EC_{50} ratio mixture of A1254 and HBCD. No significant differences.

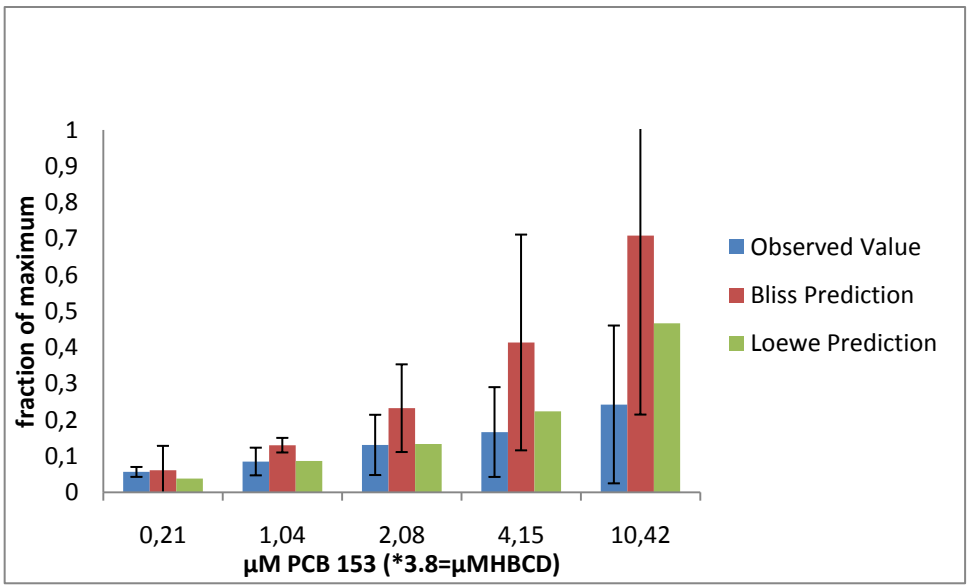


Figure 38 The experimental value and the predicted values for different concentrations of an EC_{50} ratio mixture of PCB153 and HBCD. No significant differences.

Mixtures with Bisphenol A

All the binary mixtures with Bisphenol A showed a suppressed response compared to the predictions by independent action (Fig.39-41).

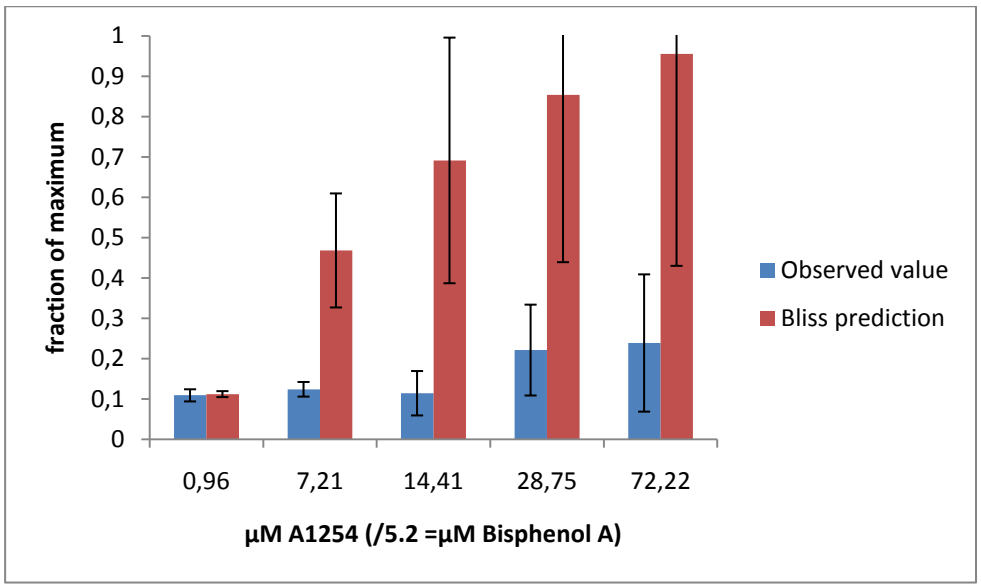


Figure 39 The experimental value and the predicted Bliss value for different concentrations of an EC_{50} ratio mixture of A1254 and Bisphenol A. Bliss Prediction vs. Observed Value: ***p<0.001

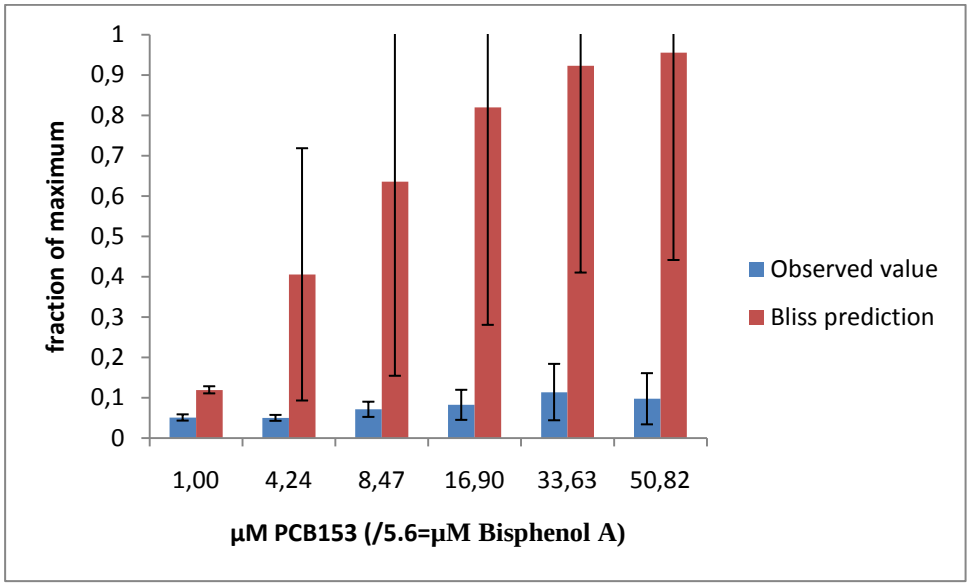


Figure 40 The experimental value and the predicted Bliss value for different concentrations of an EC_{50} ratio mixture of PCB153 and Bisphenol A. Bliss Prediction vs. Observed Value: ***p<0.001

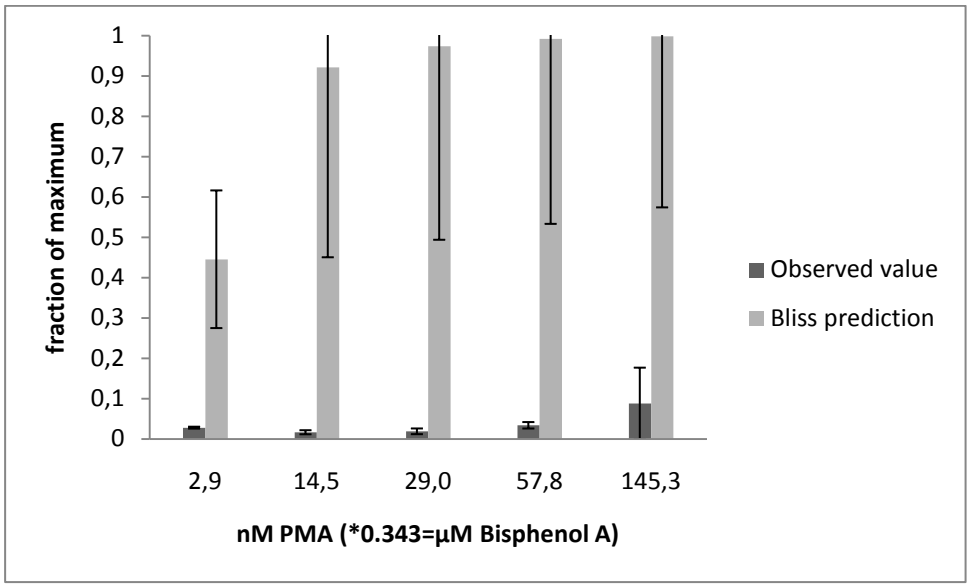


Figure 41 The experimental value and the predicted Bliss value for different concentrations of an EC_{50} ratio mixture of PMA and Bisphenol A. Bliss Prediction vs. Observed Value: ***p<0.001

4 Discussion

4.1 Whole Blood

4.1.1 Developing a Whole Blood Model for *Y.enterocolitica*

A model which investigates immune responses to different bacteria in whole blood is currently under development at FFI. *Y.enterocolitica* was chosen as a test bacterium because it has LPS similar to other gram-negative bacteria (Mandel et al., 2005). As seen in figure 13, increased concentrations of bacteria caused an increase in Tnf-α production. This made *Y.enterocolitica* a promising test candidate for the characterization of bacteria with the whole blood model.

One interesting observation was that the BHI medium, in which the bacteria were grown, induced cytokine production in blood (data not shown). This may be due to the growth factors present in the medium. BHI was replaced by PBS, a saline phosphate buffer, which had no effect on cytokine production. Another useful observation was that the amount of PBS did not seem to affect the amount of Tnf-α pr. CFU at the concentrations tested. During experiments the ratio between whole blood and PBS can be changed accordingly, so that less whole blood is necessary for each experiment.

There was a great variability between different donors in how well *Y.enterocolitica* survived in the blood. In addition, the predicted concentration of CFU did not match the counted CFU after the experiments. Therefore, the amount of *Y.enterocolitica* differed between each experiment. This made it challenging to reproduce experiments with this bacterium. Some of these differences could be explained by the processing of the cells with washing and disssolving in PBS, which may reduce the amount and the viability of the cells.

A solution for the variability between whole blood donors may be cryopreservation of blood. This would make it possible to get enough blood for several experiments from a homogenous mixture of donors. The problem with this solution is that treatment such as cryopreservation of blood may affect the results, for example by altering cytokine production (Chen et al., 2010). A solution to the inconsistent of amounts of bacteria present might be to make a culture of *Y.enterocolitica*, wash and dissolve the culture in PBS, make dilutions of the PBS-

solution to determine CFU, and kill the bacteria right after the dilutions are made. If the bacteria can be killed without destroying the membrane, the result should not strongly affect the immune response to the bacteria in whole blood. This culture can then be stored for use in several experiments. Another option would be to introduce a bacteria-static chemical. They do not kill bacteria, but they inhibit cell division and other processes in the bacteria.

4.1.2 Whole Blood Model with Environmental Toxicants

Environmental toxicants were screened at high concentrations, and all of the toxicants tested showed a low but significant induction of Tnf-α in some of the donors. Based on the screening experiment, PCB77, A1254 and HBCD were selected to evaluate dose-response relationships. In this experiment there was no significant formation of IL-1β. Even though there was significant production of Tnf-α at different doses of toxicants, there was no apparent relationship between dose and response. This can be explained by large individual variations in response to the toxicants. Errors in experimental setup, such as contamination with bacteria in the blood were also options. In principle, healthy subjects were chosen, but some may have had an infection, causing a high background cytokine level (Fig. 20-21). This might have hidden responses that were there.

PCB77 has previously been shown to induce Tnf-α production *in vitro* with doses as low as 3.4µM for 16h, but this was performed with HOVEC, which is human umbilical vascular endothelial cells (Wang et al., 2010). The difference in cell type or the fact that whole blood was used might explain the result. Whole blood contains proteins, ions and other chemicals that might interact with PCB77, making it less potent.

A1254 and PCB77 were then mixed with low concentrations of the bacterial analogs LPS and Pam3CSK4, 1ng/mL and 100ng/mL respectively. This was done to investigate modulations in the expected immune response to the bacterial analogs by the environmental toxicants. No modulations were observed. The lack of effect does not exclude the possibility that they have an immunomodulating effect on cytokine production.

4.2 Granulocytes' ROS Production

4.2.1 ROS Production after Exposure to Environmental Toxicants

The activation of respiratory burst and inhibition of phagocytosis are established biomarkers for immunotoxcitity in wildlife species (Zelikoff et al., 2000). ROS production by neutrophil granulocytes might affect cellular homeostasis by cellular signaling or gene regulation (Finkel, 1998; Allen and Tresini, 2000). Inappropriate activation of the NADPH oxidase system is associated with tissue injury and impaired ability to clear invading microorganisms (Labro, 2000). It is therefore interesting to know if environmental toxicants cause ROS formation in human neutrophil granulocytes.

With exception of Bisphenol A there seemed to be concentration-dependent ROS-production for all toxicants tested on granulocytes. PMA, which is a well-known ROS inducer in granulocytes, was used as a positive control and showed a concentration-dependent increase in ROS-formation (Fig. 23).

Bisphenol A appeared to induce a concentration-dependent reduction of the basal ROS formation as measured with DMSO (Fig. 22). This was in contrast to the results by Reistad et al. (2005) which showed, depending on the probe used, either a small increase in ROS production as a result of BPA exposure, or no effect at all. In their study, Reistad et al. used the DCFH-DA-fluorescence assay to measure ROS, which primarily detects intracellular ROS, such as H_2O_2, $OH^{-\bullet}$, $^{\bullet}NO$ and $ONOO^-$ (Freitas et al., 2009). Reistad et al. also used a lucigenin chemiluminescence assay, which gave no increase in ROS. Lucigenin detects extracellular $O_2^{-\bullet}$. The difference in results might be explained by the use of different probes or the localization of ROS formation in the cell. Luminol detects both intracellular and extracellular ROS, primarily HOCl (Myhre et al., 2003).

HBCD seemed to induce a small concentration-dependent increase in ROS production, with results significantly different from DMSO at 20µM and 50µM (Fig. 22) HBCD has previously been shown to induce oxidative stress in zebrafish embryos (Deng et al., 2009), but not in cell preparations, such as cerebellar granule cells, as measured with DCHF-DA (Reistad et al., 2006).

Both A1254 and PCB153 induced a concentration dependent increase in ROS production with results significantly different from DMSO at 5µM (Fig. 22-23). The maximum response of PCB153 was higher than the maximum response for A1254. Previously, Voie et al. (2000) showed that induction of respiratory burst in granulocytes was structure dependent. They found that a moderate size, ortho-substitution and a specific 2,4,6-substitution on one ring may produce optimal activity. Aroclor 1254 is dominated by di-*ortho*-chlorinated PCB congeners with an average of five substituted chlorines (Bedard and May, 1996). Reistad et al. (2005) also found ROS formation at 10µM PCB153 in human granulocytes with the lucigenin chemiluminescence assay, but no effect with DCFH-DA assay. This may indicate that only extracellular ROS were produced, or that $O_2^{\bullet-}$ and HOCl were the only ROS formed, which according to Freitas et al. (2009) are not detected by DCFH-DA.

Triclosan induced a small concentration-dependent increase in ROS-production with results significantly different from DMSO, for all concentrations tested (Fig. 23). It reacted differently from the other environmental toxicants, with a small ROS-production induced early in the measurements. Triclosan has previously been shown to induce oxidative stress in the earthworm *Eisenia fetida* (Lin et al., 2010).

Viability has not been investigated in this study. If the environmental toxicants caused cell death during the assay, this could cause less ROS production than expected. If for example A1254 is more cytotoxic than PCB153, this might explain the higher effect of PCB153 on ROS formation.

Reistad et al. (2005) investigated lactate dehydrogenase leakage, which is an indication of cell death, after exposure to Bisphenol A at 20µM in 5 minutes and 12 µM in 30 minutes without observing reduced viability. PCB153 was also investigated this way at 5µM with similar result (Reistad and Mariussen, 2005).

4.2.2 LPS, *Y.enterocolitica* and Environmental Toxicants.

Two approaches to investigate immunomodulating effects on an actual pathogen invasion were tested on human neutrophil granulocytes: LPS priming and bacterial invasion by *Y.enterocolitica*. LPS priming simulates a bacteria exposure where LPS is released from the infected site into the blood. Neutrophils have some TLRs so their ROS-production might be

affected by LPS. They are, in addition, known phagocytes so they were expected to produce ROS after exposure to *Y.enterocolitica*.

LPS priming of human neutrophil granulocytes seemed to cause a suppression of the ROS-formation normally induced by A1254, PAM and Triclosan (Fig. 25). LPS alone did not cause any ROS production at the concentration used for priming the cells (data not shown). LPS does not kill host cells or inhibit cellular functions. It starts a cascade of events which can lead to systemic shock and death of the host (Galanos and Freudenberg, 1993). A suppression of ROS-production could also be caused by degeneration of the cells due to incubation temperature or the time consumed by the priming process.

Y.enterocolitica seemed to affect ROS production in a concentration-dependent manner. The effect was significant when the ratio between neutrophile granulocytes and bacteria was approximately 1:1 (Fig. 26). This ratio together with increasing concentrations of A1254 gave no significant results on ROS production compared to the same concentrations of A1254 alone (Fig. 27). The same was true for the positive control with PMA (10nM) (data not shown). With PCB153, however, induction of ROS in human neutrophil granulocytes seemed to be suppressed by the presence of *Y.enterocolitica* (Fig. 28-29). The reason for the difference in response between the two PCB mixtures is not obvious and deserves a more thorough investigation. It may be due to influence on cell viability and congener-specific effects.

As for PCB153, Triclosan's induction of ROS in human neutrophil granulocytes also seemed to be suppressed by the presence of *Y.enterocolitica* (Fig.29). Here a two-way ANOVA indicated that the interaction between *Y.enterocolitica* and the dose of Triclosan varied significantly. Figure 29 illustrates that a higher dose of Triclosan had a different effect to the treatment than a low dose. The antimicrobial activity of Triclosan (Fang et al., 2010), might explain the reduced ROS production. At low concentrations Triclosan inhibits the lipid production, and at high concentrations it will disrupt the membrane of the bacteria, without perforating the membrane (Villalain et al., 2001)(see section 1.3.4). Depending on the morphology of dead bacteria, phagocytosis of *Y.enterocolitica* might still occur and induce ROS formation. The lower response to Triclosan at higher concentrations can be due to inactivation of some of the Triclosan due to the antimicrobial activity, or it could indicate a shift in response towards fighting an infection.

The effect of toxicants in combination with bacteria could not be predicted with the Loewe or the Bliss model. The dose-response curve of *Y.enterocolitica* lacked a maximum effect (Fig.26) which is necessary to create an asymetrical Hill-shape curve (section 2.3.2). With use of the simple effect summation, which is only valid for chemicals with a linear dose response relationship (Berenbaum, 1989), the results indicated that the bacteria had versatile effects on toxicants. Depending on where the effects are on the dose-response curve, the effects could have been over- or underestimated with this approach.

4.2.3 Mixture Effects in Granulocytes

Binary mixtures were made between Triclosan, HBCD and Bisphenol A and PMA and PCBs. It was difficult to decide if concentration addition or independent action would apply, because the mechanism of effect for some of the chemicals was unknown. Therefore, two models, Loewe concentration addition and Bliss independent action, were used to predict mixture effects. PMA and PCBs have known mechanisms of effects; if either the Loewe or the Bliss predictions were applicable this could give an indication of the mechanism of Triclosan, HBCD or Bisphenol A.

Triclosan seemed to follow neither Bliss nor Loewe at high concentrations in binary mixtures (Fig. 33-36). At high concentrations of mixtures with A1254, PMA and PCB153 the effect was antagonistic. Mixtures with PMA seemed to follow the Bliss prediction or even be synergistic at low concentrations.

In the binary mixture with HBCD and Triclosan there was a tendency towards more than an additive effect or at least more independent action than concentration addition. This was, however, not significant due to variation between donors (Fig. 34). HBCD in mixture with either A1254 or PCB153 seemed to follow the Loewe model of additivity (Fig. 37-38) and this might indicate that HBCD works by mechanisms similar to ortho-chlorinated PCBs.

Bisphenol A clearly reacts in a different manner than PMA, A1254 and PCB153. Therefore independent action was the only model used for predictions of binary mixtures with Bisphenol A. The mixtures were antagonistic compared to the model, especially the mixture with PMA (Fig. 41). The antagonistic effect might be caused by underestimation of Bisphenol A's slope because of the experimental set-up. The slope of Bisphenol A seemed to reach a

plateau at 5µM (Fig. 22) and this might indicate that it had supressed all ROS formation at that concentration.

In Binary mixtures with Bisphenol A and either A1254 or PCB153, Bisphenol A seemed to supress most of the ROS the PCBs produced. Bisphenol A mixed with PCBs had no negative slope as it had alone, but it seemed to suppress the response of PCBs (Fig. 30). With PMA, on the other hand, the slope went further down (not significantly) before it started to rise again (Fig. 30), indicating another kind of effect.

As seen in figure 31 and 32 the AUC, which indicates the kinetics of ROS production, changed shape for some of the mixtures compared to the individual AUC shape for the compounds. At lower concentrations of the mixtures with Triclosan and PCBs, the mixture seemed to follow the kinetics of the PCBs. At higher concentrations of triclosan mixed with PCB153 the mixture followed the kinetics of PCB153 but with suppressed effects at higher concentrations (Fig. 32). This is different from the PCB mixture with A1254. Here the triclosan kinetics seemed to control the reaction-kinetics at the highest concentration (Fig. 32).

At the lowest concentration of PMA and Triclosan, PMA's kinetics seemed to control the reaction. At higher concentrations of the mixture the reaction started early and lasted for some time, this seemed to be a combination of the reaction-kinetics of Triclosan and PMA. At the highest concentration of the mixture Triclosan seemed to control the kinetics (Fig. 32).

HBCD and Bisphenol A mixtures appeared to follow the same kinetics as the single compounds. Bisphenol A lowered the general response of A1254, PCB153 and PMA. HBCD gave the same responses, other than with Triclosan, which had kinetics that seemed to be a combination of the compounds of the binary mixture (Fig. 31). It seemed that the ROS formation was induced by several mechanisms, where one mechanism either dominated the ROS production or stopped the other mechanisms from inducing ROS. This phenomenon remains to be elucidated.

5 Future Work and Conclusion

5.1 Future Work

5.1.1 Whole Blood

The whole blood model is intended to be used on unknown pathogens in order to develop a database with a characterization of known bacteria's cytokine production. The theory is that this database can be used on unknown pathogens to compare cytokine response with known pathogens, which might indicate a similar degree of pathogenicity *in vivo*. This is uncharted territory and requires much further work.

With *Y.enterocolitica* several other cytokines, and maybe also interferons, need to be characterized; this is necessary to get enough markers for comparison. It is also necessary to investigate the incubation time of the model. One reason for this is that different cytokines have peaks at different times during the incubation period (Wang et al., 2000). Cryopreservation of blood should also be investigated. Bacteria instigate high cytokine production so the benefit of a similar blood population might be more important than some suppressed effects.

It is of interest to know if environmental toxicants affect a healthy person the same way they affect a sick person. In our study, it seems that the effect of environmental toxicants on monocytes cytokine production in whole blood is minimal. An inflammatory situation was also investigated, but PCB77 and A1254 with the bacterial analogs did not show any immunomodulating effects. Since I found nothing conclusive, this should be investigated further. It has previously been postulated that there is a link between the concentration of environmental pollutants, like PCBs, and mortality caused by different viruses in sea mammals such as seals and dolphins (Aguilar and Borrell, 1994; Olsson et al., 1994). Also, Triclosan has been shown to reduce the amount of inflammatory cytokines in inflammation caused by LPS in whole blood (Barros et al., 2010). This might be the case for other environmental toxicants such as Bisphenol A and TPPBA.

It would also be interesting to see if Triclosan affects Tnf-α production induced by *Y.enterocolitica* the same way it does LPS. To be able to compare results, a dose-response

curve for *Y.enterocolitica* should be developed. Then a concentration in the middle of the linear range could be used, to make sure that Triclosan can affect Tnf-α production in either direction.

5.1.2 Granulocytes

Environmental toxicants and bacteria affect ROS production in human neutrophil granulocytes. The effect of Bisphenol A, Triclosan and HBCD on ROS production should be investigated further. In other studies, Bisphenol A has caused a slight elevation in ROS response (Reistad et al., 2005) and it would be interesting to know if this has something to do with where the ROS are produced. Also, how the effect takes place should be investigated. In an investigation of a polymerization process with Bisphenol A, Kodoma and Fujisawa (2000) suggested that Bisphenol A scavenged radicals, this could explain why the basal DMSO level of ROS is supressed by Bisphenol A. Triclosan gives an early response in ROS production and the mechanisms involved should be investigated. The mode of action of HBCD should also be investigated as it might have similar mechanisms to ortho-chlorinated PCBs. The viability of the cells after exposure to the environmental toxicants should also be investigated.

LPS seems to reduce the effect exhibited by environmental toxicants. This should be investigated further to check if the cells' viability is affected by the priming with LPS. It is also interesting to see if these results will be the same with other environmental toxicants. This could indicate that TLR activation causes a shift in the priority of the cells away from ROS production, towards cytokine production or other responses.

Y.enterocolitica seemed to affect the effect of the ortho-chlorinated PCB153 more than A1254, indicating congener-specific effects, even though A1254 primarily contain ortho-chlorinated PCB congeners. The first step would be to investigate the viability of human neutrophil granulocytes after PCB exposure. This could indicate whether PCB153 is more cytotoxic than A1254. Also, a dose-response curve with *Y.enterocolitica* should be developed so concentration addition can be used on mixtures with *Y.enterocolitica* and ortho-chlorinated PCBs, because they both activate the NADPH oxidase system.

There was a large variation in ROS-response between donors, making it challenging to establish certain results, especially in the mixture experiments. Use of granulocytes from male donors may reduce the variation in response. Of practical reasons, blood was in our study.

sampled from female volunteers and not from male as previously performed at our institute (e.g. Reistad et al., Myhre et al., Voie et al.). It has been claimed that male blood is less influenced by hormonal changes, which may influence experiments. Whether gender is a factor that may influence ROS production in granulocytes remains, however, to be elucidated.

Mixtures of environmental toxicants are important to investigate. If the constituents of a mixture are additive or synergistic when mixed together, this could affect the risk management of these compounds. Mixtures with more constituents, similar to what we might be exposed to in different scenarios, should be investigated further. The binary mixtures studied were mainly antagonistic at higher concentrations. The kinetics observed could indicate that the concentration of the mixture decides if one or both of the constituents are involved in ROS formation. This could mean that a cell is not able to use two pathways for ROS production at maximum capacity at the same time, or that if one pathway is activated the other one is blocked. This should be investigated further.

The models predicted most of the effects of the binary mixtures poorly. The effects observed were often lower than the predicted effects, so from risk assessment perspectives this was not a problem. The luminol chemiluminescence assay on human neutrophil granulocytes may not be the perfect method for investigations of mixture effects. The luminol detects several ROS, and we do not know what ROS the constituents of the mixtures induced production of. The prediction models might work better on a lucigenin chemiluminescence assay because it only detects $O_2^{-\bullet}$ (Freitas et al., 2009). The total ROS production could be more relevant when extrapolating results from *in vitro* to *in vivo*. On the other hand, the cells were isolated, so it is not possible to conclude anything about actual effects in blood. As seen with the whole blood model, toxicants may become less potent in blood, where there are numerous constituents they could interact with.

The mixtures with HBCD and PCBs or Triclosan followed the predictions better than any of the other mixtures. HBCD and PCBs followed the Loewe model, while HBCD and Triclosan followed the Bliss model closely. It would be interesting to investigate the effects of the single constituents, with a focus on whether there are any similarities in the pathways leading to ROS formation.

5.2 Conclusion

In this thesis mixture effects between different toxicants, bacteria and bacterial analogs have been investigated on cytokine production in whole blood and ROS production in human neutrophil granulocytes.

When characterizing *Y.enterocolitica* with the whole blood model, I found a concentration-dependent increase in production of Tnf-α in whole blood. To reduce background production of Tnf-α in whole blood the bacteria should be dissolved in PBS not medium. The amount of PBS does not affect the relative production of Tnf-α. The whole blood model had too much variation between different donors to elucidate the induction of cytokine production by environmental toxicants. The results from these experiments were inconclusive.

The environmental toxicants showed potential to induce ROS in human neutrophil granulocytes. Compared to what is expected to find in blood (Thomsen et al., 2002; Knutsen et al., 2008; Kvalem et al., 2009), the concentrations used in these experiments were high. Because of this and because of the complex mechanisms involved in ROS formation it is difficult to extrapolate to *in vivo* reactions. There are, however, scenarios with lipid soluble contaminants, where animals may be exposed to extremely high concentrations of pollutants similar to what was used in this survey. The highest level of PCBs in brain is found in dead Polar sea gulls from Svalbard and ranged from approximately 3-90 µmol/kg (Gabrielsen et al., 1995). Another probable scenario is mixtures with similarly acting toxicants, here the total concentration may be comparable to the concentrations used in this survey (Rajapakse et al., 2001). Also, even though the concentration found in humans are low, they might affect humans or animals when they are vulnerable, such as during development or infections. And if starvation occurs, rapid mobilization of fat might cause a sudden increase in the toxicants' concentration in the blood (Klaassen and Watkins, 2003a).

Bacteria also affected ROS production in human neutrophil granulocytes. Therefore this was an endpoint where investigation of mixture effects between toxicants and pathogens was possible. LPS seemed to lower the response normally caused by the toxicants tested and also the response to PMA by the NADPH oxidase system. *Y.enterocolitica* seemed to have a versatile effect on the effect of different toxicants. The bacteria did not suppress the effect by A1254, as it did with PCB153 and Triclosan. This indicates that there might be differences in the reactions towards environmental toxicants depending on the state of the immune system.

A probable scenario is that the reactions of the body depend on the capability and priorities of the cells involved. The effect of environmental toxicants will vary accordingly.

The Loewe– and the Bliss models predicted the mixture effects poorly. The binary mixtures were mostly antagonistic compared to both models; exceptions were mixtures with HBCD. This could indicate that the mechanisms of action of the chemicals are too complex to predict the results with these models (Traas and Leeuwen, 2007). If one wants to predict mixture effects of environmental toxicants on the immune system, another method, like cell death assays, might be simpler and follow the models more closely. It would be interesting to know at what level of complexity the models still hold. If the models are to be used for risk assessment purposes, the level of complexity might indicate how well the results can be extrapolated to *in vivo*.

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Appendix

I. Methods

i. Environmental Toxicants and Bacterial Analogs tested on Whole Blood.

Table 7 Concentrations tested on whole blood.

Chemical	Concentrations tested	Diluent
A1254*	5, 10, 20, 50µM	DMSO
A1242	50µM	DMSO
Bisphenol A	50µM	DMSO
HBCD	5, 10, 20, 50µM	DMSO
PCB 153	50µM	DMSO
PCB 77*	0.1, 1, 5, 10 µM	DMSO
TBBPA	50µM	Metanol
Triclosan	50µM	DMSO
LPS	0.01, 0.05, 0.1, 0.5, 1, 1.5, 5, 10ng/mL	0.9%NaCl
Pam3CSK4	2, 10, 50, 100, 1000ng/mL	0.9%NaCl

*** were also mixed with 1ng/mL LPS and 100ng/mL PAM3CSK4.**

ii. Recipe for Assay Buffer A and Stop Solution

Table 8 Recipe for Assay Buffer A

Chemical	**Amount**
NaCl	8.0g
Na_2HPO_4	1.13g
KH_2PO_4	0.2g
KCl	0.2g
Distilled H_2O	→1l

Stop Solution:

2N H_2SO_4 =1M H_2SO_4

95-97% (w/w) H_2SO_4

Mw=98,08 g/mol Specific gravity= 1,84 kg/L

Molar concentration = ($1,84x10^3$ g reagent/L) x (96g H_2SO_4 /100 g reagent) x (1 mol H_2SO_4 / 98,08 g H_2SO_4) = 18,0 mol/L

To make 100 mL 1 mol/L H_2SO_4 we need: 1 mol/L x 0,1 L = 0,1 mol

Volum av 96% w/w H_2SO_4: 0,1 mol/ 18 mol/L = 0,00550L = 5,5 mL

5,5 mL 96% H_2SO_4 diluted to 100 mL with dH_2O.

iii. Environmental Toxicants tested on Granulocytes

Table 9 Concentrations used in dose-response experiments.

Chemical	Concentrations tested
A 1254*+	1, 5, 10, 20, 50µM
Bisphenol A	1, 5, 10, 20, 50µM
HBCD	1, 5, 10, 20, 50µM
PCB 153+	1, 5, 10, 20, 50µM
PMA*	1, 2.5, 5, 10, 25nM
Triclosan*+	2, 5, 10, 20, 50µM

***** indicates environmental toxicants mixed with cells primed with the bacterial analog LPS. +indicates environmental toxicants mixed with *Y.enterocolitica*.

Table 10 Concentrations of single constituents of a mixture.

Mixtures 1/2	Concentrations 1	Concentrations 2	Ratio*
Triclosan/A1254	0.9, 4.4, 8.9, 17.7, 44.4 µM	1.1, 5.6, 11.1, 22.1, 55.6 µM	1.2
Triclosan/PCB153	0.7, 2.7, 5.4, 13.4, 32.4 µM	1.0, 3.6, 7.3, 18.1, 43.7 µM	1.4
Triclosan/PMA	1.0, 5.0, 10.0, 19.9, 49.6 µM	0.7, 3.5, 7.0, 14.0, 34.8 nM	1427
Bisphenol A/A1254	0.2, 1.2, 2.8, 5.5, 13.9 µM	1.4, 7.2, 14.4, 28.7, 72.2 µM	5.2
Bisphenol A/PCB153	0.2, 0.75, 1.5, 3.0, 6.0, 9.0 µM	1.0, 4.2, 8.5, 16.9, 33.6, 50.8 µM	5.6
Bisphenol A/PMA	1.0, 5.0, 9.9, 19.8, 49.8 µM	2.9, 14.5, 29.0, 57.8, 145.3 nM	343
HBCD/A1254	0.8, 4.0, 8.0, 16.0, 40.2 µM	0.4, 2.0, 3.9, 7.8, 19.5 µM	2
HBCD/PCB153	0.8, 4.0, 7.9, 15.8, 39.6 µM	0.2, 1.0, 2.1, 4.1, 10.4 µM	3.8
HBCD/Triclosan	0.8, 4.2, 8.3, 16.6, 41.8 µM	0.3, 1.6, 3.3, 6.5, 16.4 µM	2.5

* The ratio is the approximate ratio between the two substances' concentration in the mixture, calculated as the one with the lowest concentration divided by the one with the highest concentration.

iv. Recipes for Reagents used on Granulocytes

Table 11 Recipe for Hanks' Balanced Salt Solutions (HBSS) (Invitrogen, 2005)

	Molar weight with water	gram	Solved in Xl H_2O	mL	Final concentration g/L
$CaCl*2\ H_2O$	147.02	7.351	0.05	1.258	0.185
KCl	74.56	18.640	0.1	2.146	0.4
KH_2PO_4	136.09	6.805	0.05	0.441	0.06
$MgCl2*6H_2O\ \ _2HH_2O$	203.3	10.165	0.05	0.492	0.1
$MgSO4*7H_2O$	246.48	12.324	0.05	0.406	0.1
$NaHCO_3$	84.01	2.100	0.05	8.332	0.35
$Na_2HPO_4*2H_2O$	177.99	8.900	0.5	26.968	0.06
NaCl	58.44	73.050	0.5	54.757	8
H_2O				➔	

v. Isobol Diagram

This was developed by Loewe and Muischnek (1926) to compare the EC of mixtures with predicted ECs.

In this study the EC_{50} of either the Bliss - or Loewe model would be an additivity line between the concentration of a and b that is expected to cause a 50% effect of the mixture. Then an EC_{50} is calculated based on the observed results of the mixture. The concentration of each of the compounds at the EC_{50} are the coordinates of a point; if the models hold the point should be on the additivity line. If it was below the line this would indicate synergy and above the line this would indicate antagonism.

It was not possible to calculate EC_{50} for the mixtures. One reason was the variation of effect between donors, another reason was the bell-shape of some of the dose-response curves. Therefore the results are compared by individual points instead.

II. Results

i. Growth Rate of *Y.enterocolitica*

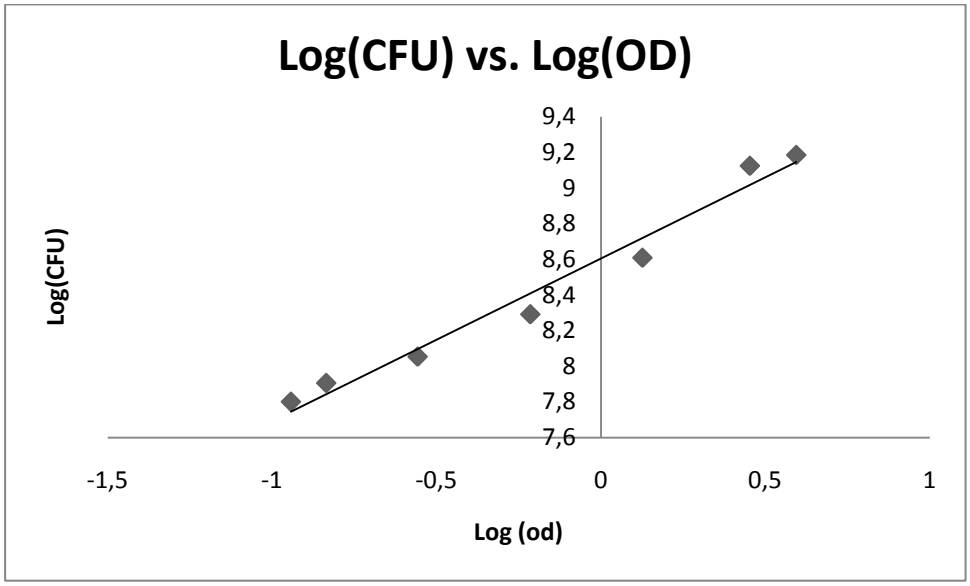

Figure 42 The relationship between Log(CFU) and Log(OD) should be linear. R=0.975, which gives a good indication that the relationship is linear.

To make sure that each experiment had the same amount of bacteria present from the start the growth rate of *Y. enterocolitica* was determined as described in section 2.4.1. This experiment was done several times, but with small changes each time. The result from the last experiment, shown in figure 40, was used as an approximate estimate for the relationship between CFU and OD (described in equation 4).

$$\text{Log CFU}=0.9111*\text{Log(OD)}+8.6042 \quad (4)$$

At every experiment with *Y. enterocolitica* CFU was counted.

ii. Confirmation of Bacteria Species:

All of the tests came up (Fig. 43) during the PCR. Different amounts of DNA may cause the difference in when the slope starts to rise.

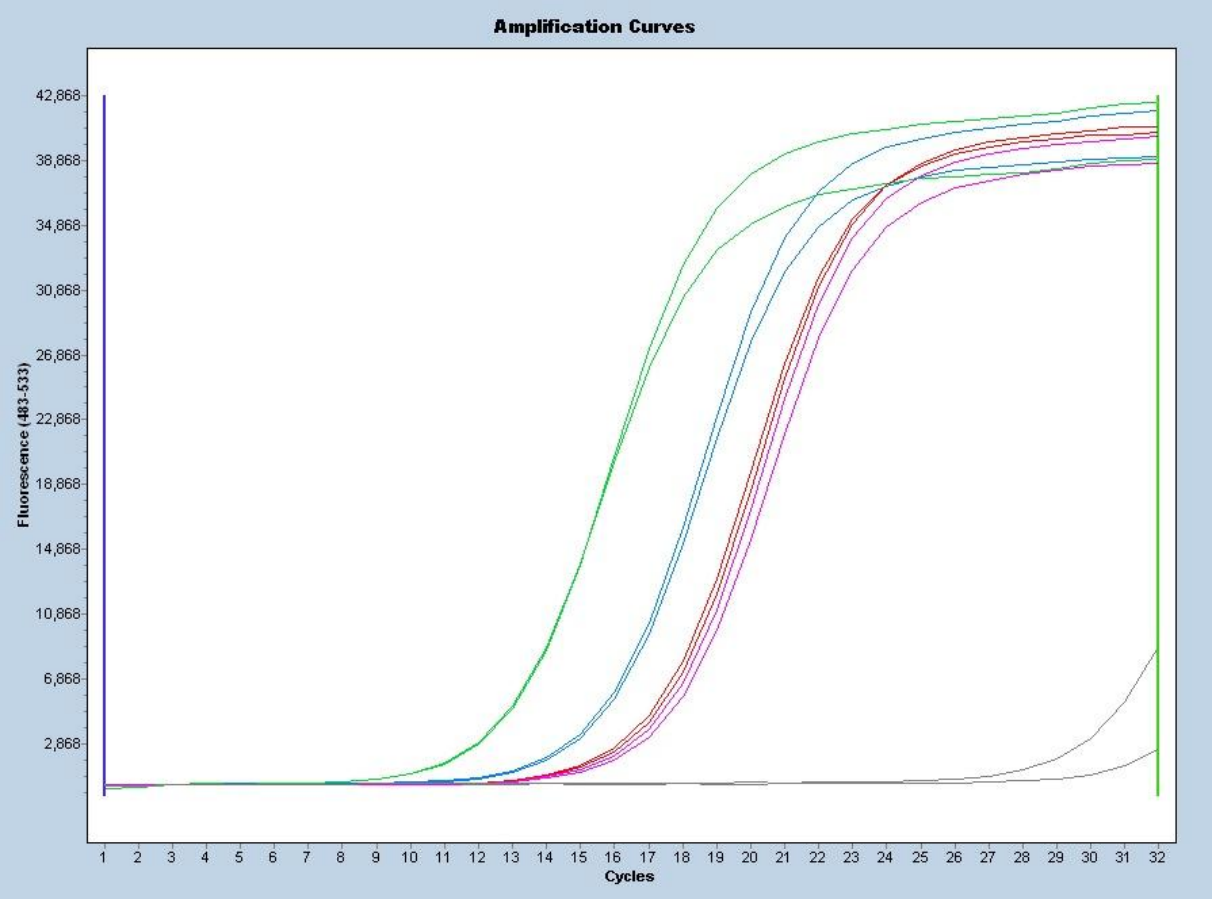

Figure 43 Amplification curves.

The similar meltingpoints indicates that they have the same ratio between C and G nucleotides in the PCR-product (Fig. 44), an indication that the PCR products are the same.

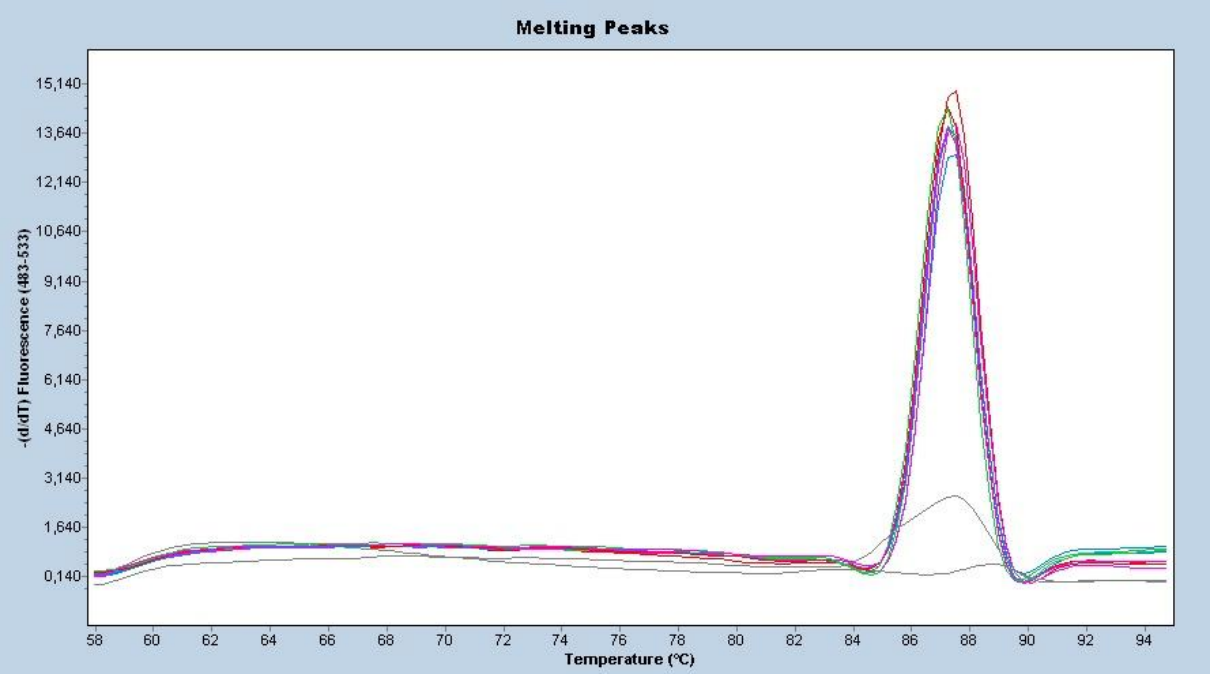

Figure 44 Melting Peaks

The final results from the sequencing were identical. After a BLAST nucleotide search and ribosome database project the results presented in figure 45 and 46 came up as the closest matches. This agrees with the Maldi-TOF experiment presented in table 11.

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
FJ717343.1	Yersinia enterocolitica subsp. enterocolitica strain ATCC 9610 16S ril	806	806	100%	0.0	100%	
EF179129.1	Yersinia enterocolitica strain CCUG 12369 16S ribosomal RNA gene, p	806	806	100%	0.0	100%	
EF179128.1	Yersinia enterocolitica strain CCUG 11291 16S ribosomal RNA gene, p	806	806	100%	0.0	100%	
EF179127.1	Yersinia enterocolitica strain CCUG 7758 16S ribosomal RNA gene, pa	806	806	100%	0.0	100%	
AF366378.1	Yersinia enterocolitica 16S ribosomal RNA gene, partial sequence	806	806	100%	0.0	100%	
Z49828.1	Y.enterocolitica gene for 16S ribosomal RNA (strain ER-3206-92)	806	806	100%	0.0	100%	
M59292.1	Yersinia enterocolitica 16S ribosomal RNA	802	802	100%	0.0	99%	
CP002246.1	Yersinia enterocolitica subsp. palearctica 105.5R(r), complete genom	800	5444	100%	0.0	99%	
FR729477.2	Yersinia enterocolitica subsp. palearctica Y11	800	5527	100%	0.0	99%	
HM007567.1	Yersinia enterocolitica strain 5 16S ribosomal RNA gene, partial sequ	800	800	100%	0.0	99%	
FJ717344.1	Yersinia enterocolitica subsp. palearctica strain DSM 13030 16S ribos	800	800	100%	0.0	99%	
EU178101.1	Yersinia enterocolitica (type 0:9) 16S ribosomal RNA gene, partial se	800	800	100%	0.0	99%	
EU538505.1	Uncultured bacterium clone nbt230h09 16S ribosomal RNA gene, part	800	800	100%	0.0	99%	
EU536404.1	Uncultured bacterium clone nbt213d05 16S ribosomal RNA gene, part	800	800	100%	0.0	99%	

Figure 45 Blast Results: *Y.enterocolitica* ATCC9610 is the top result.

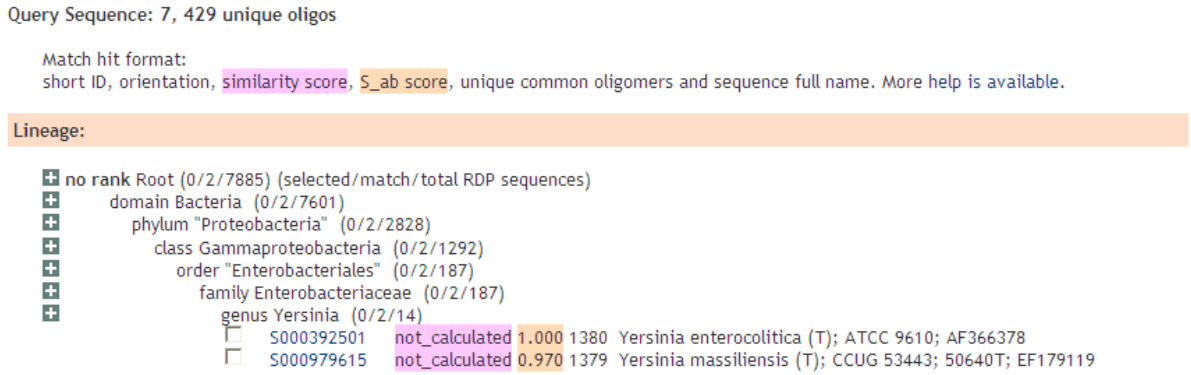

Figure 46 Ribosome Database Project results: *Y.enterocolitica* ATCC9610 is the top result.

The result from MALDI-TOF gave a score value of 2.406, 2.300-3.000, indicates highly probable species identification (table 11). The result was *Y.enterocolitica* (serovar O8) ATCC 9610.

Table 12 Results from the Maldi confirmation of bacteria

Analyte Name	Analyte ID	Organism (best match)	Score Value	Organism (second best match)	Score Value
YE (+++)		Yersnina Enterocolitica (serovar O8) ATCC 9610T THL e Enterocolitica	2.406	Yersiniae Enterocolitica	2.231